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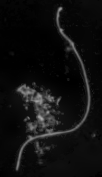

Canadian Journal of Research

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VOL. 27, SEC. E. OCTOBER, 1949 NUMBER 5

THE EFFECT OF LIVER EXTRACT PREPARATION ON BONE MARROW EXPLANTS[1]

BY K. E. ARMBRUST AND H. D. BETT

Abstract

A study has been made of the effect of various concentrations of several liver extract preparations on the migration of cells, chiefly leucocytes, from explants of guinea pig bone marrow. The migration has been shown to be stimulated by an extract of liver, which shows incomplete activity in the treatment of pernicious anemia. Two clinically active fractions have been shown to be inactive in the stimulation of cell migration. The migration of cells from explants cannot be used under the conditions described as a test for antipernicious anemia activity of liver extract preparations.

Introduction

Many attempts have been made to develop a laboratory procedure for the determination of the potency of liver extracts intended for the treatment of pernicious anemia. Overbeek and associates (8, 9) described a method involving the migration of cells from explants of guinea pig bone marrow. When these explants were cultured in a coagulum of plasma, cells, chiefly leucocytes, migrated to form a reasonably sharply defined zone of migration. The addition of a clinically active liver extract, in a series of dilutions, was said to stimulate the migration. The effect of the liver extract on the area of migration was used as an index of the potency.

Pace and Fisher (10) followed this work and claimed that the clinical potency of the extracts could be related to the dilution of the extracts at which maximal migration of the cells from bone marrow explants occurred. A curve was constructed to show this relationship. Young and Bett (13), however, could not show this relationship, and reported that no definite relationship between bone marrow response and antipernicious anemia activity could be demonstrated with this method. Subsequent work by Armbrust and Bett (2) indicated that a relationship could be established by a modification of the method used by Pace and Fisher. At this time it was stated that the bone marrow explant test might be useful in determining whether or not a liver extract had antipernicious anemia activity.

During the past year, eight fractions of liver extract have been tested clinically and by the bone marrow test. Four of these gave positive results in both tests. Tests on the remaining four fractions have indicated that it

[1] *Manuscript received May 7, 1949.*

Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ont.

is possible to separate bone marrow migration factor from antipernicious anemia substance. These are the results that are presented in this paper.

Method

The method used for the bone marrow test was essentially as previously described (2). The preparation of the bone marrow was improved to some extent by cutting on plastic rather than wood. This made it possible to get pieces more nearly even in size and more regular in shape. Another refinement in the method was to place the dishes, from which the bone marrow was to be distributed, around the circumference of a 15 cm. Petri dish. The cover of this dish had a hole drilled through it 1 cm. from the edge and 1 cm. in diameter. The bone marrow could be reached by inserting a pipette through this hole. Since the top could be turned to each dish, it was unnecessary to remove the cover each time a piece was to be placed in a culture dish. In this way the marrow was protected from contamination. Six pieces of bone marrow were placed in each culture dish as shown in Fig. 1. In each test of a liver extract two dishes were used at each of two concentrations, namely 0.16×10^{-3} and 0.33×10^{-3} ml. of liver extract per ml. of medium. In each test two dishes, to which no liver extract had been added, were also used.

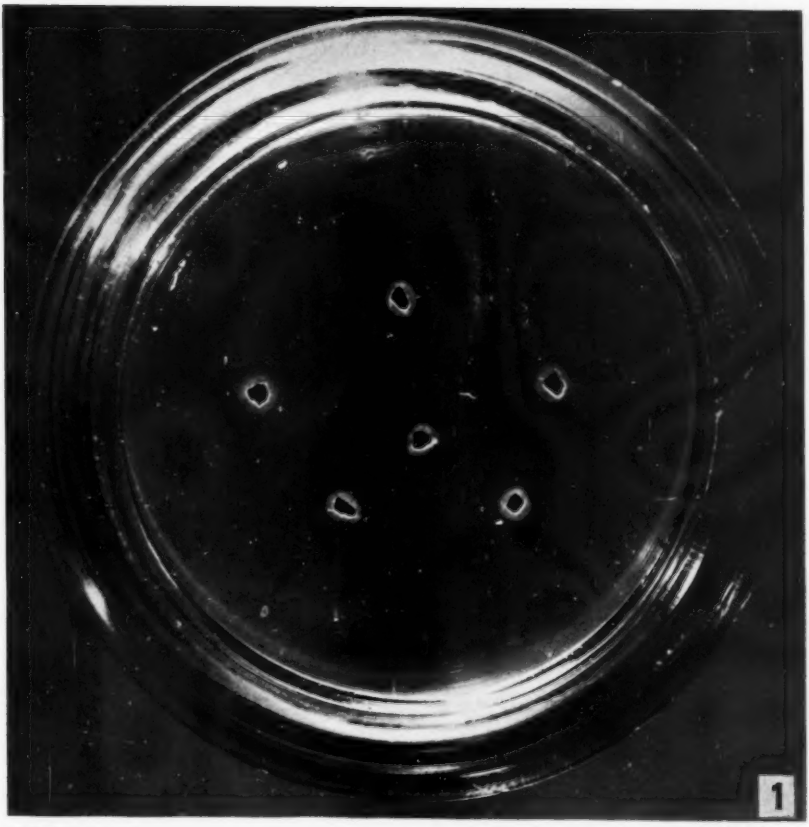
The area of migration for each piece of bone marrow was measured at 30 diameters. Figs. 2 and 3 show two pieces at a magnification of 25 diameters. The results reported in this paper are for three separate tests. The average area of migration in the case of the control and at each concentration is therefore based upon results with 36 pieces of bone marrow. The temperature of the room in which the test was conducted was about 25° C.

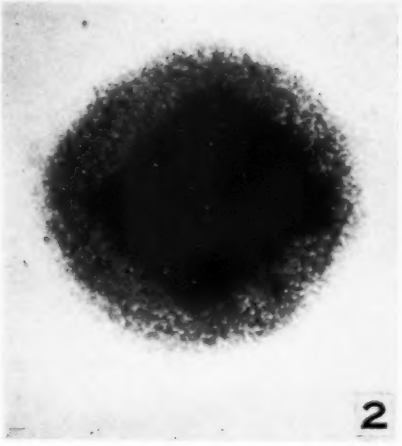
Each ml. of liver extract fraction was prepared from the same weight of liver and the clinically active solutions contained 15 units per cc. when tested on pernicious anemia patients.

Results

Table I shows the results of tests of four fractions of liver extract. The standard deviation of a single observation was 30 units. The unit refers to the smallest squared area (4 sq. mm.) on graph paper.

Fraction 137 was considered on clinical trial to be incompletely active in the treatment of pernicious anemia. Clinically active material was fractionated into two parts to produce Fractions 139 and 140. Further purification of Fraction 140 provided Fraction 143. Both 140 and 143 were shown to be clinically active extracts. In the bone marrow test, 143 showed no response to a change in concentration of the extract in the medium and 140 showed less response than 137 or 139. The results on 139, 140, and 143 were obtained in simultaneous tests. Since fraction 140 produced a maximal clinical response, it appears unlikely that any antipernicious anemia activity can be present in Fraction 139. However, this fraction has not been subjected to clinical test. These results indicate a separation of antipernicious anemia substance and migration factor.





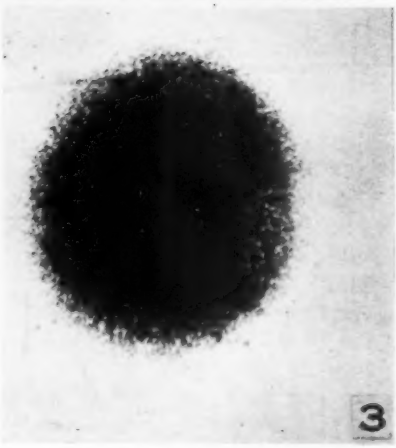


FIG. 1. *Bone marrow explants after incubation, 2×.*
FIGS. 2 AND 3. *Two explants showing area of migration as it would be seen on projection, 25×.*

TABLE I

EXTENT OF MIGRATION PER PIECE OF BONE MARROW

(The number of squares, having an area of 4 sq. mm. each, traced on graph paper)

Liver extract	Ml. of liver extract per ml. of medium		
	0.0 ml.	0.16×10^{-3} ml.	0.33×10^{-3} ml.
	Extent of migration		
Fraction 137	100 squares	125 squares	165 squares
Fraction 139	80	130	165
Fraction 140	90	100	110
Fraction 143	65	70	60

During the course of this investigation, tests were carried out in which the migration, at the various concentrations of liver extract, was very great. Tests on the serum indicated the presence of a factor that stimulated the migration. At normal concentration of serum in the medium, the average migration was 120 units; at one-half concentration, 108 units; and at one-quarter concentration, 83 units.

It has not been unusual to have a reduction in migration when a concentration of 0.3×10^{-3} ml. of active liver extract per ml. of medium has been exceeded. This was not the case with Fraction 139. The reduction in response with cruder fractions supports the evidence of previous workers (9, 10) for peak values in areas of migration at certain concentrations. Thus it seems that factors responsible for inhibiting the migration may have been removed in the purification of Fraction 139.

Clinical Results

The clinical testing of these extracts was carried out in the Department of Medicine at the Toronto General Hospital.

Extract 137

This extract was administered to a male patient 69 years of age, with an initial erythrocyte count of 1.3 millions per cu. mm. and a haemoglobin of 31% (Sahli-Leitz Haemoglobinometer 15.6 gm. = 100%). One cubic centimeter of extract was given and 1 cc. after two weeks had elapsed. During a 30-day period the erythrocyte level did not exceed 2.0 million per cu. mm. and the haemoglobin did not rise higher than 48%. A marked reticulocytosis (33%) appeared on the sixth day after the first injection and a lesser response (19%) on the sixth day after the second injection. Subsequent treatment with an active liver extract (15 units per cc.) used routinely in the clinic, produced the usual increase in erythrocytes and a third reticulocyte response (13%) on the sixth day after injection. The active extract was given in ½ cc. doses each week for three weeks. At the end of this period the erythrocyte level had reached 3.5 million per cu. mm. and the haemoglobin 75%.

Extract 140

This extract was administered to a male patient, 51 years of age, with an initial erythrocyte count of 2.1 million per cu. mm. and a haemoglobin of 56%. One-half cubic centimeter of extract was given each week for four weeks. On the sixth day after the first injection a reticulocyte peak of 24.6% had been reached. At the end of the test period the erythrocyte count was 4.0 million per cu. mm. and the haemoglobin 80%.

Extract 143

This extract produced a striking initial haematopoietic response. Its power to maintain pernicious anemia patients is under investigation.

The extract was administered to a female patient, 53 years of age, with an initial erythrocyte count of 2.7 million per cu. mm. and a haemoglobin of 64%. One cubic centimeter of the extract was given followed by 1 cc. after two weeks had elapsed. A reticulocyte peak of 17.8% was reached on the fifth day after the initial injection. A good subjective response was reported at this time. At the end of the four week test period the erythrocyte count was 4.2 million per cu. mm. and the haemoglobin 92%.

A second patient was treated with this extract. A male patient, 53 years of age, with an initial erythrocyte count of 1.1 million per cu. mm., and a haemoglobin of 27%, received 0.5 cc. doses of this extract at intervals of one week. Five days after the first injection the reticulocytes had reached a peak of 46.4%. At the end of the first week the erythrocyte count was 2.4 million per cu. mm. and the haemoglobin 58%. At the end of four weeks the erythrocyte count was 4.0 million per cu. mm. and the haemoglobin 72%. This patient has been maintained on 1 cc. of this extract every two weeks from December 1947 to the present.

Discussion

Although it has been shown that migration of cells in bone marrow explants is not an indication of the antianemia value of liver extracts, the work has not progressed to the point where a statement can be made as to the nature of the substance responsible for the stimulation of the migration.

It would appear that the migration phenomenon is something apart from the erythropoietic response observed in patients with macrocytic anemia when treated with liver extract (7), folic acid (1, 4, 5, 6, 11), or vitamin B_{12} (11, 12). Patients treated with liver extract from which the migration factor had been removed showed the usual clinical response. From our results it is apparent that the migration is not stimulated by the specific antipernicious anemia substances contained in liver extract. If we assume that vitamin B_{12} is the antipernicious anemia factor, then it is not the migration factor. The folic acid content of the extract with which we started is low (less than 1.0 μgm. per ml.). Preliminary work has shown that this substance is required at a higher concentration than this, before any significant stimulation of the bone marrow *in vitro* takes place. Berry *et al.* (3) have shown the

presence of a factor, in certain preparations of liver extract and vitamin B_1, that increased the neutrophile count in patients suffering from malnutrition. Since this appears to be a direct effect on the delivery of granulocytes from the bone marrow it may be of the same nature as the substance with which we are concerned.

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ELECTRON MICROSCOPY OF TYPHUS RICKETTSIAE[1]

By C. E. Van Rooyen and G. D. Scott

Abstract

This report contains an account of a systematic study on the morphology of five rickettsiae pathogenic to man, investigated with the electron microscope. The work has bearings on the biology, antigenic structure, and taxonomy of the rickettsiae.

Technique

We have used an RCA type E.M.U. model electron microscope. Well washed suspensions of rickettsiae were inoculated on formvar covered 200 gauge stainless steel wire mesh mounts. The latter were repeatedly washed with water, allowed to dry, and fixed with osmic acid vapor.

All preparations were examined under the microscope before and after shadowcasting.

Epidemic Typhus

A (Brienl) strain of *R. prowazeki* was cultivated in the yolk sac membrane of the embryonated egg and rickettsiae were separated by extraction with ether, followed by differential centrifugalization and washing in saline according to the method of Craigie (1).

Unshadowcast mounts revealed structures of coccobacillary shape exhibiting numerous internal masses and granules of varying electronic density. The whole appeared to be surrounded by an envelope previously described in unshadowcast pictures published by Plotz, Smadel, Anderson, and Chambers (3). Mounts were next shadowcast with chromium and measurements showed that rickettsiae varied from 0.5 μ to 2.0 μ in length, 0.3 μ to 0.7 μ in breadth, with an average size of 1.1 μ by 0.5 μ.

The latter findings (see Fig. 1) suggest the presence of an outer area of low electronic density analogous to a bacterial envelope. *R. prowazeki* is composed internally of structurally and electronically dense masses surrounded by apparently fluid substance. During the process of drying the periphery of the organism collapses leaving the central masses intact and projecting through the surface (as indicated from the contour of shadows) so as to convey the appearance of an electronically translucent periphery or envelope. The relationship of these two constituent parts of the rickettsia is somewhat analogous to that of a fluid ectoplasm and a granular endoplasm. Appearances rather similar to these have been reported in preparations of vaccinia virus treated with molar sodium chloride and shadowcast with gold by Dawson and McFarlane (2). Several elongated forms were observed showing central constriction suggestive of the beginning of binary fission.

[1] *Manuscript received May 12, 1949.*

Contribution from Connaught Medical Research Laboratories, University of Toronto, Toronto, Ont. and McLennan Laboratory, Department of Physics, University of Toronto.

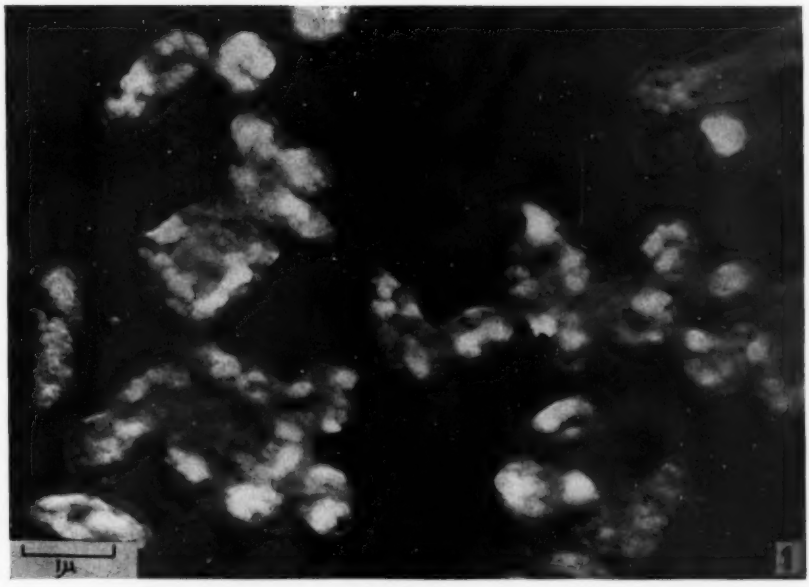

FIG. 1. *R. prowazeki.* ×20,000
FIG. 2. *R. mooseri.* ×20,000

All specimens shown have been shadowcast with chromium; the shadow to height ratio is approximately 4 : 1.

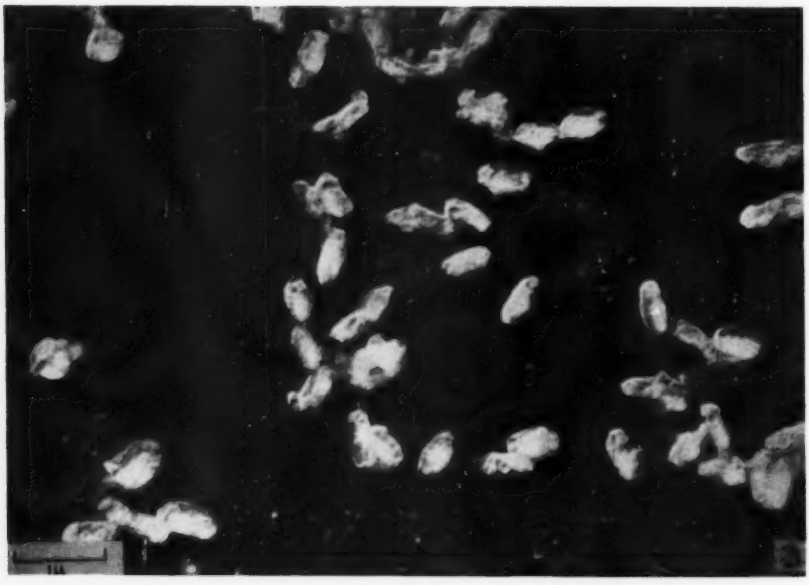

FIG. 3. *R. burneti.* ×20,000
FIG. 4. *R. burneti.* ×45,000

All specimens shown have been shadowcast with chromium; the shadow to height ratio is approximately 4 : 1.

Murine Typhus

The Wilmington strain of *R. mooseri*, grown on the yolk sac and concentrated by the manner already described, was used. Unshadowed pictures suggested the presence of more internal structure in comparison with *R. prowazeki*. In size *R. mooseri* varied from 0.7 to 1.3 μ in length, 0.35 to 0.6 μ in breadth, with an average size of 1.0 μ by 0.45 μ.

The peripheral clear area surrounding the center of the rickettsia, which we call an "envelope", appears to fit more tightly than in *R. prowazeki*; also the central masses seem to be more granular and evenly distributed throughout the organism (see Fig. 2). Individual rickettsiae showed large bipolar electronically dense masses, concentrated at each end. Evidence of binary fission was also observed.

Although *R. mooseri* (Wilmington) shows slightly less variation in size, being on the average slightly smaller than *R. prowazeki* (Brienl), we doubt whether these rickettsiae can be differentiated with certainty by electron microscopy unless the looseness of the envelope be accepted as a criterion.

Q. Fever

A concentrated suspension of *R. burneti* (Nine mile strain) was supplied by courtesy of Dr. R. L. Gauld, Army Medical School, Washington. The material was rewashed with ether and concentrated by differential centrifugalization.

Unshadowed photographs showed little detail, but after shadowing with chromium considerable structure was revealed. *R. burneti* was found to measure 0.6 μ to 0.9 μ in length, and 0.25 μ to 0.4 μ in breadth, with average size of 0.73 by 0.32 μ (see Fig. 3). Greater uniformity in size range was observed than in the case of *R. prowazeki* and *R. mooseri*. The distribution of internal structure showed considerable regularity in arrangement (see Fig. 4). The latter consisted of circular or oval shaped bodies about 120 mμ in length, 50 mμ in width. Numerous extracellular particles of about 100 mμ in diameter (see Fig. 4) were also noticed and these may have been extruded from ruptured rickettsiae. The envelope was not so noticeable and when present appeared to fit closely over the central mass.

Rickettsialpox

A strain of *R. akari*, received through courtesy of Dr. R. J. Huebner, U.S. Public Health Service, was propagated in the yolk sac of embryonated eggs and a washed suspension of rickettsiae was obtained as previously. Thereafter electron microscope mounts were prepared and pictures secured before and after shadowcasting.

R. akari showed relatively little size variation, the average being 0.9 to 1.4 μ by 0.6 μ. The envelope was evident and electronically dense masses within the organism were distributed throughout (see Fig. 5).

Rocky Mountain Spotted Fever

Photographs of *R. rickettsi* were also obtained. Antigen prepared from the "R" strain isolated from *D. andersoni* ticks provided by Dr. R. R. Parker and D. B. Lackman, of the Rocky Mountain spotted fever laboratory, Hamilton, Mont. was employed. Material was rewashed with ether and rickettsiae separated by differential centrifugalization in saline prior to use. Results showed that the average measurements of *R. rickettsi* were 1.2 μ by 0.6 μ.

In view of recent serological evidence that has indicated the antigenic relationship of *R. akari* to *R. rickettsi*, a comparison is of interest in that these two organisms also resemble one another closely (see Figs. 5 and 6).

Discussion

Three aspects of the biology of rickettsiae arise. Previously from unshadowcast photographs of the rickettsiae of epidemic, murine, Rocky Mountain spotted fever, and Q. fever, Plotz *et al.* (3) concluded that it was not possible to distinguish one variety from the other. Our results reveal after shadowcasting that differences do exist in the average sizes and structure of five strains of rickettsiae studied. Secondly, it has been shown that rickettsiae, cultivated in the yolk sac of the embryonated egg and extracted by washing with ethyl ether followed by differential centrifugation (Craigie (1)), yield preparations that may be readily photographed with the electron microscope. There is no obvious evidence to suggest that ether (at low temperature) exerts a destructive effect on the outer covering or envelope of rickettsiae.

In their studies on the nature of the soluble antigen from typhus rickettsiae, Shepard and Wyckoff (5) concluded that so-called soluble antigen of typhus consisted of submicroscopic particles of capsular substance. We have noticed what probably are these same particles but our results show that they are derived from the central body of the rickettsia and not the capsule.

The third issue, arising out of this research, raises the important question of the relationship of the rickettsiae to the bacteria on the one hand and the viruses in general and psittacosis in particular.

During the course of studies on variola vaccinia virus, van Rooyen and Scott (4), despite careful search, failed to find clearcut or convincing signs that virus elementary bodies divided by binary fission. The present work on the rickettsiae suggests that it is occasionally possible to photograph rickettsiae showing central constriction analogous to bacterial binary fission. Much further research is necessary before these points can be clarified but we believe that the rickettsiae show morphological resemblances more closely related to the bacteria than the viruses. The morphological relationship of the rickettsiae to the psittacosis group of viruses is in course of investigation.

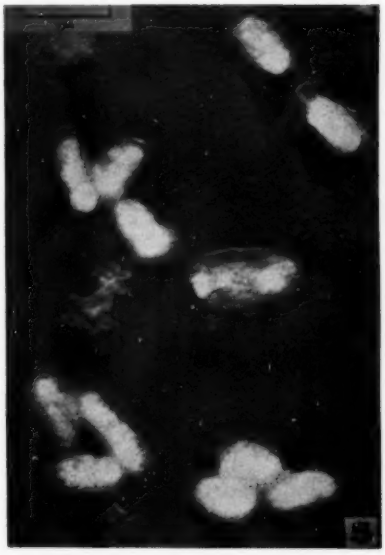

FIG. 5. *R. akari.* ×20,000
FIG. 6. *R. rickettsi.* ×20,000

All specimens shown have been shadowcast with chromium; the shadow to height ratio is approximately 4 : 1.

Conclusions

(1) Electron microscope photographs of *R. prowazeki*, *R. mooseri*, *R. rickettsi*, *R. akari*, and *R. burneti* have revealed certain differences in size and other morphological features.

(2) *R. burneti* has revealed internal structure of a pattern more striking than in other rickettsiae examined.

(3) *R. rickettsi* and *R. akari* appear to be morphologically similar.

(4) Structurally, the rickettsiae resemble the bacteria more closely than the viruses.

(5) Evidence of binary fission has been reported in rickettsiae.

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Our thanks are due to Mr. D. R. Welch for the care and patience exercised in the preparation of illustrations, also to Miss Margaret Ranney for considerable technical assistance.

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THE DETECTION AND ASSAY OF HYALURONIDASE BY MEANS OF MUCOID STREPTOCOCCI[1]

By R. G. E. Murray and R. H. Pearce

Abstract

A method is presented by which hyaluronidase may be detected by observing its action on the colony characteristics of mucoid strains of *Streptococcus pyogenes* and certain strains of *Streptococcus* of Lancefield Group C growing on agar. This effect is due to destruction of the capsules of these organisms and is caused by hyaluronidase from all sources tried. Bacteria are tested for hyaluronidase production by streaking on seeded plates; fluids are tested by using assay cylinders or filter paper disks.

The method is convenient and has the advantage of detecting small concentrations of the enzyme, to a limit of between 0.001 and 0.0001 turbidity reduction units per ml. The test may be elaborated to give a means of biological assay that may be used where there are substances in the medium or diluting fluid that interfere with assays by other methods.

Provided the colonies do not autolyze during the 12 to 18 hr. required for the test, all mucoid *S. pyogenes* strains are suitable. However, among the Group C strains one was found with a capsule not destroyed by hyaluronidase, indicating a separate capsular type.

In testing certain genera for hyaluronidase production, notably *Streptococcus* and *Micrococcus*, antibiotic activity may interfere with the test. These inhibitory strains are relatively infrequent.

Introduction

The biological importance of the mucolytic enzyme, hyaluronidase, is well known because of its relationship to the "spreading factor", and has been reviewed thoroughly by Duran-Reynals (3) and Meyer (13).

The work of Seastone (19, 20), Kendall *et al.* (8), and Meyer *et al.* (14) has shown that the capsules of mucoid *Streptococcus pyogenes* and Group C streptococci consist of a complex polysaccharide chemically indistinguishable from hyaluronic acid. The nature of this capsular polysaccharide was further elucidated by McClean (10, 11) and Hirst (7) who demonstrated that hyaluronidase from testis and from the leech was capable of decapsulating completely these strains of mucoid streptococci.

One of us observed that the colony form of mucoid *S. pyogenes* is altered when growing in the neighborhood of colonies of *Micrococcus pyogenes* var. *aureus*, *Diplococcus pneumoniae*, and other hyaluronidase-producing organisms. This suggested a possible test and assay method for hyaluronidase using methods similar to those employed for the detection and assay of antibiotics, with mucoid streptococci as the indicator of enzyme activity.

[1] *Manuscript received March 28, 1949.*

Contribution from the Department of Bacteriology and Immunology and the Department of Pathological Chemistry, University of Western Ontario, London, Ont. Presented in part at the meeting of the Laboratory Section, Canadian Public Health Association, Montreal, December, 1946 (15).

Materials

The Medium

The medium used for maintaining cultures and for the test is made as follows:

Proteose peptone No. 2 (Difco)	0.5 gm.
Proteose peptone No. 3 (Difco)	0.5 gm.
Sodium chloride	0.65 gm.
Agar (granulated, Agar Products Ltd.)	1.2 gm.
Papain broth (1) (pH 7.2)	add to 100 ml.

Four ml. of fresh, sterile, defibrinated sheep blood is added to each 100 ml. of the medium after autoclaving at 121° C. for 20 min. Thirty ml. of the medium in a 10 cm. plate provides a sufficient depth for the maximal growth of the streptococci.

The choice of a papain digest broth may be fortunate since Rogers (16) showed that the hyaluronidase production of organisms was enhanced by a similar digest although different supplements were used.

The Substrate Organisms

A Lancefield Group A, type 9, *S. pyogenes* ("M. Str. 9"), was used for the majority of these experiments. Other strains, including members of the Lancefield Group C streptococci, were found to be satisfactory. The choice of suitable strains for this test is discussed in a later section of this paper. A number of the strains have been maintained for a period of over two years without change of character.

Methods

To detect hyaluronidase production by an organism, mucoid streptococci are spread on a plate to give confluent growth at the top and closely spaced but discrete colonies at the bottom. The organism under test is then streaked from top to bottom of the plate to give a confluent line of growth (see Fig. 1).

To detect hyaluronidase in a fluid, the fluid is placed in a penicillin assay cylinder or taken up by a filter paper disk, which is then placed on the surface of the seeded medium. A confluent growth of the mucoid streptococci over the entire surface of the plate is preferred. This may be achieved either by flooding the plate with a broth culture, aspirating the excess and drying, or more simply by spreading a streak of inoculum evenly over the surface of the plate (see Fig. 3).

The plates are incubated at 37° C. for 14 to 18 hr. Suitable strains of mucoid streptococci give good colony morphology under aerobic or anaerobic conditions, with lowered oxygen or increased carbon dioxide tension. Consequently, the conditions most suited to the growth and hyaluronidase production of organisms being tested may be chosen. However, for the testing of fluids, the most consistent results were obtained very simply in a "candle jar" (a desiccator kept in the incubator in which a candle is left burning when the jar is closed).

Standard Solutions of Hyaluronidase

Enzyme preparations were standardized by means of the turbidity reduction method of assay (9, 17), which is based on the turbidity that is produced by low concentrations of hyaluronic acid in the presence of serum at pH 4.2. One turbidity reduction unit (T.R.U.) is defined as that concentration of the enzyme which, under certain standardized conditions, will reduce the turbidity produced by 0.2 mgm. per ml. of sodium hyaluronate to that produced by 0.1 mgm. The hyaluronidase used as reference during this investigation was a partially purified extract of bovine testes containing approximately 10 T.R.U. per mgm. For use the enzyme was dissolved in normal saline.

Observations

The Effect of Hyaluronidase on Colonial Morphology

A streak of *M. pyogenes* is made with a small loop from top to bottom of a plate seeded with a mucoid *Streptococcus*. After 16 hr. incubation the streak is a confluent line about 3 to 4 mm. across, and the *Streptococcus* colonies are very mucoid. Around the streak there is a zone about 20 mm. across in which the colony morphology of the streptococcus is definitely altered (Fig. 1).

For 2 to 3 mm. on each side of the streak the colonies are small, high convex, and the surface has lost the glossy appearance of the mucoid colonies. At the outer margin of this zone the colonies flatten out and become larger flat disks on the surface of the medium, covering almost the same area as the normal mucoid colony. The boundary of this zone is very sharp, so much so that colonies are often seen to be cut in half: one half flattened and the other mucoid. In the region of confluent growth (not shown clearly in the figure) the boundary of the outer zone is sharp and resembles a step down from a plateau of mucoid growth to a lower level of less glossy growth. The inner zone previously described is seen as a narrow region of rougher, almost matt surface right up to the edge of the streak. The same picture was given when other strains of *M. pyogenes*, virulent strains of *Diplococcus pneumoniae*, certain strains of haemolytic *Streptococcus* of various groups, and other organisms were tested as set forth in a later section. Ultrafiltrates of broth cultures of *M. pyogenes* and of *D. pneumoniae*, readjusted to pH 7.0 or not, gave a similar effect when placed in assay cylinders. By the turbidity reduction method these preparations were shown to depolymerize hyaluronic acid.

A partially purified bovine testicular extract known to contain 10 T.R.U. of enzyme per mgm. was used to show that hyaluronidase produced this effect. Enzyme solutions made up in 1 to 4 mgm. per ml. concentrations in saline were put into penicillin assay cylinders on the surface of seeded plates. The testicular enzyme gave results exactly similar to those obtained with *M. pyogenes* (Fig. 2).

Colonies subcultured from either zone gave normal mucoid growth on fresh plates as did the mucoid colonies. Smears from areas of normal growth, and

PLATE I

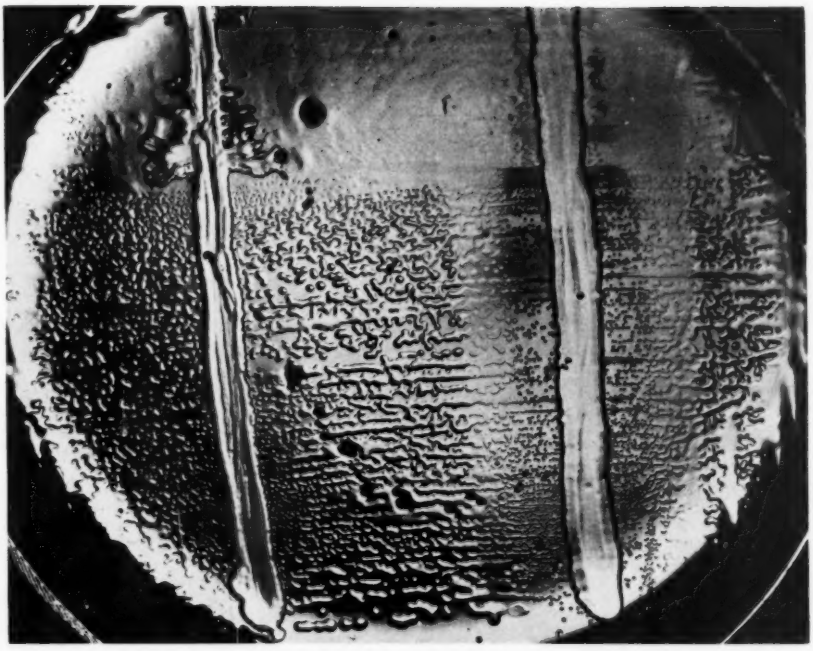

FIG. 1. *A photograph of a Petri dish spread with M. Str. 9 and streaked with two Micrococcus strains. The streak on the right is Micrococcus pyogenes var. aureus surrounded by the hyaluronidase effect. The streak on the left is a white Micrococcus sp. and does not show any effect.*

PLATE II

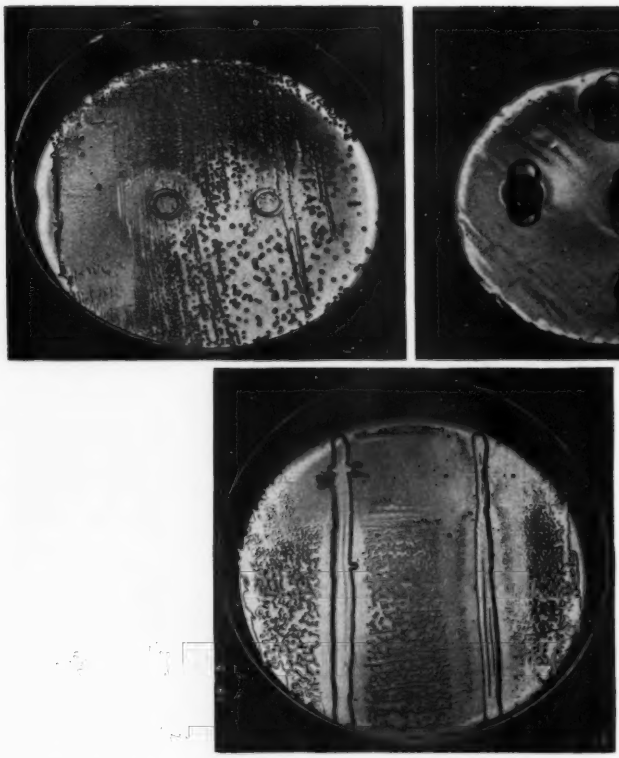

FIG. 2. *A photograph showing the effect of bovine testicular hyaluronidase on M. Str. 9. The cylinders contained fluid with approximately 1 T.R.U. per ml. of enzyme. The two zones may be seen, especially at the left.*

FIG. 3. *A photograph to show the effect of hyaluronidase on a confluent growth of M. Str. 9. This is evident around the upper and center cylinders.*

FIG. 4. *A photograph showing the inhibition of M. Str. 9 by two strains of Micrococcus pyogenes. The streak on the left shows a pure inhibition while that on the right shows inhibition with a superimposed hyaluronidase effect.*

from each of the two zones were examined for the size of capsules using the India ink method. Little difference was seen between organisms from the normal colony and the outer flattened zone, although the capsules seemed a little smaller in the latter. However, capsules of organisms from the inner (small colony) zone were absent or were definitely smaller when present. No differences were seen in the bacterial cells from the different zones. These observations were true for both the streak plates and the assay cylinder plates.

Neither saline nor phosphate buffers of pH values of 5, 6, 7, and 8 reproduced the effects seen with hyaluronidase preparations. Minor variations in the pH of fluids tested may have little effect because of the considerable intrinsic buffering power of the medium through which the tested fluid must diffuse. The ability to produce the effect is lost by heating the enzyme preparation to 100° C. Ascorbic acid, which shows definite spreading activity *in vivo*, does not show any flattening effect on colonies of mucoid streptococci in concentrations from 0.01 to 1.0% tested in the same manner as enzyme preparations.

That it is not an inhibition of the growth of streptococci can be shown by seeding a plate with substrate organisms and placing an absorbent disk (13 mm. diameter) saturated with an enzyme preparation of 1 T.R.U. per ml. After 12 hr. incubation, reading is made of the zone about the disk and an identical disk of the same enzyme concentration is placed on another portion of the plate. Readings are taken at intervals thereafter. A typical flattening effect is obtained and results are shown graphically in Fig. 5. The fact that,

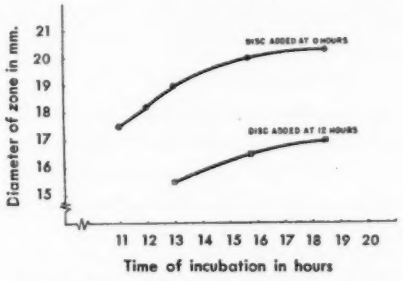

FIG. 5. *The effect of hyaluronidase when applied at different stages of growth.*

for the lower curve, the effect is not prevented because the mucoid colonies are already formed helps to substantiate the assumption that hyaluronidase acts on the capsules. The zone is smaller due presumably to the increased substrate concentration and the diffusion time. In addition, hyaluronidase preparations do not affect the growth of nonmucoid *S. pyogenes* of the same types as the mucoid strains employed.

The Effect of Various Dilutions of an Enzyme Preparation

Serial dilutions of a standardized enzyme preparation were made in saline and pipetted into penicillin assay cylinders on plates seeded to give confluent growth of the mucoid streptococcus. Following incubation, the overall

diameter of the effect was measured with vernier calipers as accurately as possible. Above a certain concentration of enzyme (ca. 1 T.R.U. per ml.) the size of the effect was constant or increased only very little whereas below that concentration the diameter of the effect decreased very rapidly. A representative experiment is shown graphically in Fig. 6. Since, in nearly

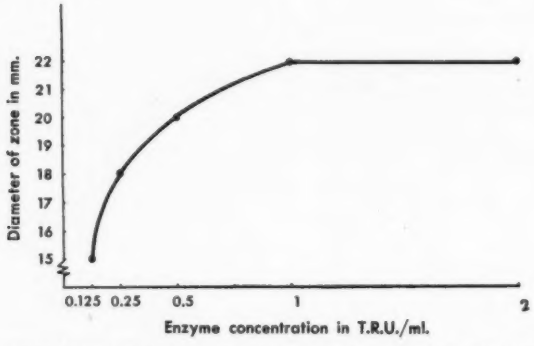

FIG. 6. *The relationship of the concentration of hyaluronidase to the diameter of the effect.*

all cases, there is a very sharp transition from the flattened zone to the normal mucoid growth this measurement may be made with reasonable accuracy. Plates were spread for confluent growth of the streptococci because of the ease of measurement of the sharply outlined zone; the appearance is shown in Fig. 3.

The Detection of Low Concentrations of Enzyme

In seven replicates, using assay cylinders (on separate batches of medium and using different lots of enzyme), 0.001 T.R.U. per ml. produced an observable effect in each case; 0.0005 T.R.U. per ml. produced an observable effect in five cases and 0.0001 T.R.U. per ml. produced an observable effect in three cases. Using filter paper disks ($\frac{1}{2}$ in. in diameter) the lowest detectable concentrations of enzyme were 10 to 100 times greater than those detected using assay cylinders in parallel tests. For tests with low enzyme concentration the assay cylinders are the most satisfactory.

The rate of diffusion of hyaluronidase, and the growth rate and the capsular stability of the mucoid streptococci have great influence on the sensitivity of the test, especially when trying to detect low concentrations of hyaluronidase. Diffusion of the enzyme under the conditions of these experiments reaches a maximum after approximately 18 hr. (Fig. 5) in the incubator. On the other hand the colonies of mucoid streptococci tend to collapse after 12 to 24 hr. incubation, varying with the strain, and after this collapse reading of the test is impossible. The strain (M. Str. 9) that we have selected for these tests remains in good mucoid state for at least 18 hr. However, with enzyme concentrations less than 0.01 T.R.U. per ml., more reliable results may be

obtained by placing the seeded plate, with the cylinders filled and in place, in the refrigerator for six hours before incubating so that diffusion may take place.

Use as a Biological Assay

From examination of the curves obtained by plotting the diameter of the effect against the concentration of enzyme as in Fig. 6, it seemed likely that a biological assay might be developed on the same basis as plate assays for penicillin and other antibiotics. It was found that the curves could be made linear if plotted on semilog paper, as shown in Fig. 7. This linear relationship applies only to concentrations of hyaluronidase of 1 T.R.U. per ml. or less.

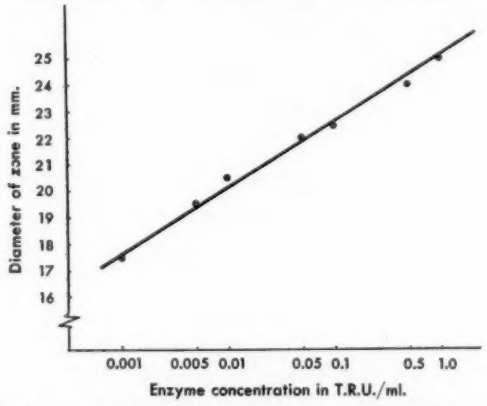

FIG. 7. *The linear curve obtained when the diameter of the effect and the enzyme concentration are plotted on semilog paper.*

Since it is found, as with other plate assays, that the diameter of effect for a given enzyme concentration varies in separate tests it is impossible to use a standard curve as reference for the assay. A standardized enzyme is used to provide points of reference for each assay. An example of an attempt at assay is given graphically in Fig. 8. In this graph the "unknown" has been assigned an arbitrary position. It is essential to such an assay that the slopes of the curves for "unknown" and "standard" enzyme preparations be as nearly identical as possible. As can be seen in Fig. 8 the parallelism is reasonably good. Knowing the dilution factors for the "unknown" the assay can be made by direct projection on the graph.

The work done on this assay is only adequate to show that the method is feasible. It would probably be worthwhile only for enzyme concentrations in the range of 1.0 to 0.005 T.R.U. per ml. and, for improvement of the accuracy, would require an even layer of medium on a flat surface large enough to take all the cylinders or disks required for both "unknown" and "standard".

Application to Tissue Extracts

The qualitative test is useful for detecting hyaluronidase activity in tissues and fractions of tissue extracts. A block of tissue placed on the seeded

medium shows little or no hyaluronidase activity. But extracts of tissue, such as testis, ground in a Latapie Mill or in a mortar with sand using a small volume of fluid, can be tested by the described techniques. The method was

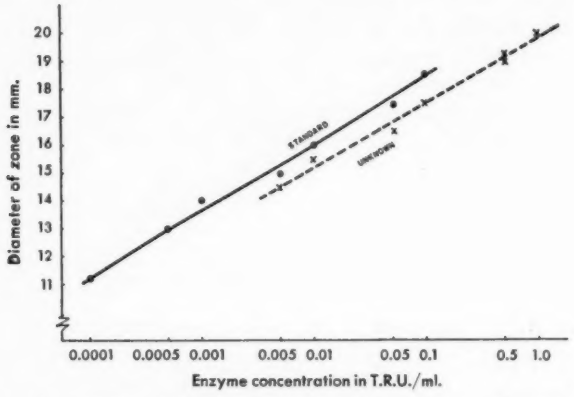

FIG. 8. *An example of the biological assay of hyaluronidase. The unknown is given an arbitrary position in the graph and is referred to the curve of a standard enzyme preparation.*

used successfully to follow activity in preparations from human, bovine, and rat testes. Whether enzyme inhibitors, such as those described by Haas (5) and others, would interfere with the testing of tissues and tissue fluids has not been investigated. However, trials were made with four hyaluronidase inhibitors (hyaluronic acid derivatives) described by Hadidian and Pirie (6) and also with heparin in the diluent fluid. These slowed the viscosity reduction of hyaluronate in the viscosimeter from two to five times. Using the plate test, no significant differences were observed between the controls and solutions containing the inhibitor, possibly because of the time relationship involved in incubation of plates for 12 to 18 hr. and of reversal due to the elevated substrate concentration.

Application to Bacteria

Using the streak plate test an extensive survey of organisms could be carried out in short time, though no such attempt was made here. The plates may be read after 12 to 14 hr. incubation and are good after an average "overnight" incubation if a suitable mucoid *Streptococcus* strain is used. The zone of flattening of colonies is always well defined and rarely comes so close to the streak that the result must be recorded as doubtful. This makes the test simple and certainly not laborious. In view of the sensitivity of the test and the tolerance of the mucoid streptococci for varied gaseous conditions, it would seem to be a suitable method for determining whether an organism does or does not produce hyaluronidase.

A survey of available strains, most of them freshly isolated, is shown in Table I. It can be seen that, in general, the organisms known to produce

TABLE I

A SURVEY OF ORGANISMS FOR HYALURONIDASE PRODUCTION USING THE PLATE TEST

Organisms tested	No. of strains tested	Not producing hyaluronidase	Producing hyaluronidase	Doubtful
Streptococcus pyogenes (Group A)	17	5	12	0
Streptococcus (haemolytic ? Group A)	6	2	4	0
Streptococcus pyogenes (mucoid)	12	12	0	0
Streptococcus (Group B)	2	1	1	0
Streptococcus (Group C, human)	1	0	1	0
Streptococcus (Group C, "animal pyogenes")	7	7	0	0
Streptococcus (minute haemolytic, Group G)	1	0	1	0
Streptococcus (Group H)	1	0	1	0
Streptococcus faecalis	2	1	0	1
Streptococcus (*viridans* group)	7	6	1	0
Diplococcus pneumoniae	12	0	12	0
Micrococcus pyogenes	113	0	112	1
Micrococcus sp. (coagulase negative)	6	6	0	0
Klebsiella pneumoniae Type A	1	0	0	1
Type B	1	1	0	0
Corynebacterium diphtheriae	6	6	0	0
Clostridium welchii	1	0	1	0
Clostridium histolyticum	1	1	0	0
Clostridium septique	1	0	1	0

hyaluronidase give the characteristic changes on mucoid streptococci. The streptococci (45 strains from Groups A, B, and C) show the known variability of hyaluronidase production as exemplified in the work of Crowley (2). In Crowley's series, hyaluronidase production in Group A was restricted to types 4 and 22 to which we may add types 9 and 19, although not all our strains were typed. Sherwood (22) has used this plate test on a large number of strains of streptococci of various groups and found "complete correlation" with viscosimetric and mucin clot prevention methods of detecting hyaluronidase. The capsulated virulent pneumococci were all active, but one type II strain dissociated to rough and lost the ability to produce the enzyme. Among the micrococci the positive correlation of hyaluronidase production and the production of plasma coagulase shown by Schwerbacher *et al.* (18) is supported, with an indication that the correlation may be better than they reported.

An antibiotic effect produced by certain organisms may interfere, but in our experience the frequency of these strains is low. Sherwood (21, 22) has described representatives from nine Lancefield Groups that produce an antibiotic inhibition of one or more other strains of β-haemolytic streptococci including the M. Str. 9 used in our tests. Three of Sherwood's strains were found to inhibit all of our strains of mucoid Group A and Group C streptococci to varying degrees. However, on those where inhibition was very slight, there was no evidence of a superimposed hyaluronidase effect. Out of the 113 strains of *M. pyogenes* examined, three were found to have an inhibitory

effect. Of these three, two produced a hyaluronidase effect much wider than the inhibition (see Fig. 4), which was easily recognized. The remaining strain did not inhibit two other strains of mucoid *S. pyogenes* and on these it gave a very narrow and doubtful zone and is recorded as such in Table I. These inhibitors are being investigated.

Qualitative tests may be carried out with broth cultures, although with less convenience, and using the projected method of biological assay it is possible to get a figure equivalent to T.R.U. per ml. for the medium in which the organism is grown. All cells must be removed from the medium or their growth will give altered results. Using Corning fritted glass U.F. filters we have obtained good results providing at least 10 ml. is filtered, after preliminary clarification by centrifugation. Lower activity is obtained with smaller amounts, presumably because of some adsorption to the filter. These tests have been hard to control because of the effect on the turbidity reduction assay of various constituents (? peptones) of the complex media required for adequate growth and enzyme production by the pneumococci that were used in these experiments.

To find out whether some media in common use would effect the test when used as diluents, an enzyme preparation of 5.0 T.R.U. per ml. was diluted 10 times with each medium under test and placed in three assay cylinders (Table II).

TABLE II

Diluent	Mean zone diameter of 3 cylinders, mm.
1. Normal saline	18.3
2. Peptone water	18.7
3. Papain broth	18.5
4. Dextrose broth	16.7
5. Brewers medium	15.5
6. Litmus milk	15.0

There is a significant difference between the two main groups, 1, 2, and 3 and 5 and 6; with 4 in an intermediate position. These differences are probably not due to inhibition of the enzyme but, much more likely, to the presence of fermentable carbohydrate stimulating the growth of the mucoid *Streptococcus*. This stimulation was evident in the controls and, causing an increase in the amount of substrate to be hydrolyzed, may be expected to give the deviation of readings observed. The medium in use must, therefore, be considered as possibly deflecting the sensitivity of the test especially if it contains fermentable carbohydrate.

The Choice of Mucoid Strains as Substrate Organisms

Strains of *S. pyogenes* with an obviously mucoid appearance are of comparative rarity in clinical bacteriology. They have a large colony form that is rather watery in appearance, and, when fished, have a very sticky mucinous

character. On examining a number of strains thought to be mucoid on primary isolation the sticky colony grades almost insensibly to colonies of the same order of appearance but of a pasty character when fished, although they all have capsules of varying size at some time during growth. Many of the latter strains were found to produce hyaluronidase whereas none of the 12 truly mucoid strains produced the enzyme. Among the 12 strains, four were unsuitable as substrate organisms because the colonies flatten too quickly or because of a tendency to dissociate. Our original strain, M. Str. 9, proved the most satisfactory together with a probably identical strain, 1175 A9. Stability of the mucoid colony and exuberant growth are the most necessary characteristics of suitable strains for this test.

Strains of *Streptococcus* ("animal pyogenes") Lancefield Group C have a very persistent mucoid state on incubation and should be, theoretically, suitable for this test. Seven strains were tried. None of them produce the hyaluronidase effect on the suitable *S. pyogenes* strains. Six strains were affected by both bacterial and testicular hyaluronidases, though some were more suitable than others because of more exuberant growth. Strain 34406 C, however, despite its grouping and excellent capsulation is not affected by bovine or human testicular extract nor by different strains of known hyaluronidase producing bacteria (Group A streptococci, pneumococci, and micrococci). This is evidence that there may be two types of mucoid strains—with capsular polysaccharides sensitive and not sensitive to hyaluronidase.

Discussion

The methods used for the detection and assay of hyaluronidase (13) are beset with many difficulties and disadvantages. Chemical methods are not yet sufficiently specific, simple, and sensitive enough to be of value for routine use. Physical methods involving viscosity and turbidity require rigidly controlled conditions, special apparatus, and the isolation of hyaluronate. The mucin clot prevention test is sensitive but often at variance, quantitatively, when referred to other means of assay (12). Biological methods using skin diffusion are expensive, subject to variability, and other "spreading" substances may be present in preparations. The physical methods are the most used and the most reliable. However, for some work they are unsatisfactory because they are not particularly sensitive to low concentrations of enzyme.

A test, based on the decapsulation of Group C streptococci by hyaluronidase, was reported recently by Fulton, Marcus, and Robinson (4). The degree of decapsulation is determined by observing changes in the packed cell volume of a suspension treated with various concentrations of enzyme. The method has the advantage of speed and is sensitive down to 0.01 viscosity reducing units* per ml., but has the disadvantage of requiring considerable manipulation.

The biological method described in this paper provides a qualitative test that requires for its success only the provision of adequate growth conditions

* *Viscosity reducing unit equivalent to two turbidity reducing units.*

for a mucoid *Streptococcus*. The fact that it is very sensitive and will detect a minimum concentration of between 0.001 and 0.0001 T.R.U. per ml. is a point in its favor. A minimum of manipulation is required. It is also relatively unaffected by the presence of extraneous substances such as those found in media. The method can be applied as a simple test for the production of hyaluronidase by growing bacteria and to the detection of the enzyme in both crude and purified tissue extracts. Although it is feasible it does not seem necessary to elaborate the test as a means of biological assay until a need arises.

Acknowledgments

The authors are indebted to Dr. G. G. Kalz (McGill University, Montreal), Dr. E. T. Bynoe (Laboratory of Hygiene, Ottawa), and Dr. R. M. Pike (Southwestern Medical College, Dallas, Texas) for the cultures they supplied. Dr. Noble P. Sherwood (University of Kansas, Lawrence, Kans.) was most generous in supplying inhibitory strains of streptococci and kindly undertook a trial of the method. Dr. N. W. Pirie (Rothamsted Experimental Station, England) was kind enough to provide samples of hyaluronidase inhibitors. The Schering Corporation, Plainfield, N.J., supplied generous amounts of purified hyaluronidase, through the courtesy of Dr. Erwin Schwenk. We would like also to thank many colleagues, especially Dr. I. N. Asheshov, for aid and counsel. The photographs used in the paper were taken by Mr. W. Austin of the Art Service, University of Western Ontario.

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THE MICROCOLORIMETRIC DETERMINATION OF INORGANIC PHOSPHATE IN PLASMA AND URINE[1]

I. QUANTITATIVE INVESTIGATION OF PRESENT COMMON PROCEDURES
II. A SENSITIVE AND STABLE METHOD FOR PLASMA, SERUM, AND URINE

By John R. Polley[2]

Abstract

A quantitative investigation of the common colorimetric procedures used for determining inorganic phosphate was made to determine the factors affecting the sensitivity and color stability. A method is presented that is accurate to within ±2% and is sufficiently sensitive to give quantitative color development with less than 1γ of phosphorus. The color produced is quite stable for colorimetry. The method has been adapted to the determination of phosphorus in 0.1 or 0.02 ml. of plasma, serum, or urine or in the protein-free filtrate residue from the determination of sodium, potassium, calcium, and chloride in 0.4 ml. of plasma.

Phosphorus is present in the blood to the extent of about 40 mgm. %, mainly in the forms of lipid phosphorus (12 mgm. %), inorganic phosphate (5 mgm. %), and organic acid-soluble phosphate esters (25 mgm. %). For the quantitative determination of these different forms, they are first separated on the basis of their physical chemical properties. The phosphorus content in each case is then determined by first converting the phosphorus to inorganic phosphate by ashing, where necessary.

A method for the microdetermination of sodium, potassium, calcium, and chloride in a single plasma sample was published recently (8). In this procedure, 0.4 ml. of plasma is added to 3.5 ml. of distilled water, followed by the addition of 1.0 ml. of 15% trichloroacetic acid. After centrifuging, about 4.7 ml. of protein-free filtrate is obtained by decanting the supernatant liquid, 1.0 ml. aliquots being used for each of the above analyses. It was proposed to devise a method for the determination of inorganic phosphate on 0.5 ml. of the remaining filtrate.

The differences between the various methods for phosphorus determination lie in their treatment of the inorganic phosphate solutions. Most procedures in present use are variations of two older methods that differed in the reducing agent used: 1-amino-2-naphthol-4-sulphonic acid in the method of Fiske and Subbarow (4), stannous chloride in the method of Kuttner and Cohen (6).

The use of 1-amino-2-naphthol-4-sulphonic acid was proposed by Fiske and Subbarow as a reducing agent instead of the hydroquinone previously used by Bell and Doisy (1). They found that this reagent rapidly reduced the phosphomolybdic acid at room temperature and imparted the advantages that the intensity of the blue color produced was less affected by pH differences in the

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Contribution from the Department of Medical Research, University of Western Ontario, London, Canada.

[2] *This work was done during the tenure of a Life Insurance Medical Research Fellowship.*

solutions and was more stable for colorimetry than that given by hydroquinone. Roe and Whitmore (10) simplified the method and the preparation of reagents.

Stannous chloride as reducing agent was introduced by Kuttner and Cohen (6), the method being modified later by Kuttner and Lichtenstein (7), for the specific reduction of the phosphomolybdic acid. They found that the intensity of the blue colored solution was about 10 times that of the Fiske and Subbarow method and was proportional to the phosphorus content. However, it was found that with the molybdic acid – stannous chloride reagent, an excess of acid decreased color development, whereas an excess of sodium molybdate increased the color intensity but also increased the color in the reagent blank. Using a modified preparation of the reagents, Shinowara, Jones, and Reinhart (11) found that by reducing the acidity of the protein-free filtrate, the effect of trichloroacetic acid on the diminution of color development described by Bodansky (3) was completely negated. The method has the disadvantage that the color intensity changes with time.

In 1938, Berenblum and Chain (2) devised a method in which the reducible phosphomolybdic acid is extracted from the aqueous solution with isobutyl alcohol and then reduced with stannous chloride. This method is less sensitive to changes in acidity, concentration of reagents, and interfering substances than those previously described.

Kitson and Mellon (5) described a method for the determination of phosphorus in steels using ammonium vanadate rather than the previous reducing agents. A yellow colored solution of molybdivanadophosphoric acid is formed, the color intensity of which is determined colorimetrically as a measure of the phosphorus content. This method has been adapted to the determination of serum phosphate (12).

From these introductory remarks it can be seen that the stannous chloride method is considered to have the advantage of increased sensitivity but the color intensity changes with time and is somewhat pH dependent, whereas the use of 1-amino-2-naphthol-4-sulphonic acid or ammonium vanadate gives increased color stability but the color is less intense and so less sensitive.

A quantitative comparison of these different methods was made using the following experimental procedure and phosphate standards. The various experimental conditions such as pH and relative reagent concentrations were investigated in an attempt to develop a method possessing the optimum of color intensity and stability. In each case, the reagents were freshly prepared according to the authors' directions. The instrument used was an Evelyn photoelectric colorimeter with a 660 filter for the blue solutions.

Experimental

A tentative model of an experimental procedure was set up as follows:

(1) To 5 ml. of 5% trichloroacetic acid add 0.5 ml. of protein-free filtrate.

(2) Add 0.5 ml. of molybdate reagent.

(3) Add 0.5 ml. of reducing agent in five minutes.

1-Amino-2-naphthol-4-sulphonic acid as Reducing Agent

The reagents were prepared as outlined by Roe and Whitmore (10); 2.5% ammonium molybdate (Step 2 above), 250 mgm. % 1-amino-2-naphthol-4-sulphonic acid in sulphite–bisulphite solution as reducing agent (Step 3 above). The variables investigated were (1) ammonium molybdate concentration, (2) reducing agent concentration, (3) effect of trichloroacetic acid.

The effect of the concentration of the ammonium molybdate solution was investigated by preparing calibration curves between galvanometer readings and solutions of known phosphorus content over a range of concentrations of ammonium molybdate. In each case, a reagent blank was prepared and the instrument set to 100 with the blank in place (set to 0 with instruments having a semilogarithmic scale). The colored solutions were read from time to time to observe the stability. In Fig. 1 is shown the calibration curve obtained over a range of ammonium molybdate concentrations from 0.5 to 2.5%, the readings being made after five minutes. It can be seen that, over this

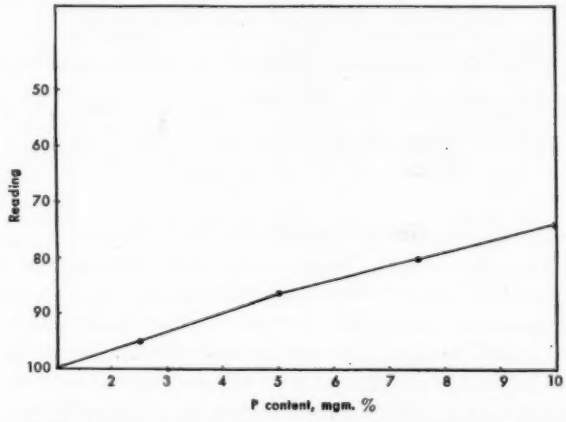

FIG. 1. *Calibration curve using Fiske and Subbarow method over a range of ammonium molybdate concentrations from 0.5 to 2.5%.*

range, the calibration curve is independent of the concentration of the ammonium molybdate solution. Below 0.5%, color fails to develop quantitatively. A disadvantage not apparent in Fig. 1 is shown in Fig. 2, which shows the effect of increasing ammonium molybdate concentration on the stability of the color produced with a given phosphate standard (2.5 mgm. % P) over a period of two hours. As the concentration of the ammonium molybdate solution is increased, the intensity of the color produced is increased but the color stability is decreased; the color intensity in all tubes, including the reagent blanks, increases and continues to increase with time. Despite this, up to a concentration of 2.5%, the calibration curve remains almost constant over a period of two hours when the reagent blank is set to 100 for each reading.

Above 2.5% the color is so intense and unstable that it is impossible to construct an accurate calibration curve. At 0.5%, the reagent blank is colorless and the calibration curve is stable. Increasing the concentration of the

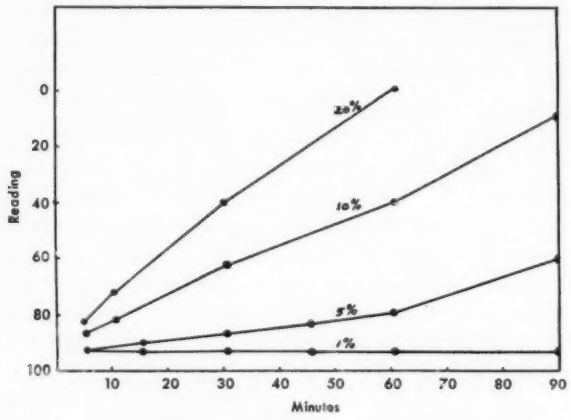

FIG. 2. *Effect of increasing ammonium molybdate concentration on color stability.*

ammonium molybdate solution above 0.5% does not increase the sensitivity of the method, since the increased color produced in the phosphate standards is negated by the increased color in the reagent blank and the color becomes more unstable.

In a similar manner, the effect of varying the concentration of the reducing agent was studied. It was found that as the concentration was increased from 50 to 550 mgm. %, the intensity of the color produced with a given phosphate standard increased, but so did the color in the reagent blank. When the correction for the reagent blank was made in each case, all tubes gave almost identical readings, showing that the net color intensity is not sensitive to changes in the reducing agent concentration over the tenfold range tested. When 10% ammonium molybdate was used instead of the 2.5%, the color intensity increased as the concentration of the reducing agent was decreased but in all cases the color was very unstable.

The effect of trichloroacetic acid was studied in a similar manner by varying the concentration of it used in Step 1. It was found that, over a range of concentration from 0.5 to 15%, the calibration curves obtained were almost identical. Likewise, the color stability was not affected by the amount of trichloroacetic acid present.

Briefly, this method is quite insensitive to trichloroacetic acid, relatively independent of the reducing reagent concentration over the range of 50 to 500 mgm. % tested, and independent of the ammonium molybdate concentration between 0.5 and 2.5% but seriously affected above this.

Stannous Chloride Method

The reagents were freshly prepared as outlined by Shinowara, Jones, and Reinhart (11); 3% molybdic acid—1 part of 7.5% sodium molybdate + 1 part of 10 *N* sulphuric acid (Step 2), 0.07% stannous chloride (Step 3).

The effect of varying the concentration of the molybdic acid was studied as before by preparing calibration curves over a range of molybdic acid concentrations. The tubes were read from time to time as a check on the color stability. It was found that over a range of concentrations from 1 to 10%, the calibration curves obtained at a given time interval after the addition of the last reagent did not differ by more than three galvanometer divisions (0.3 mgm. % P) at any point. Over a period of two hours, the color intensity faded slightly so that all the calibration curves showed a small drift. This drift, however, was not greater than five scale divisions (0.5 mgm. % P). Between 30 and 60 min. the color was almost completely stable. The intensity of the color produced with a given phosphate standard rose to a maximum with 1% molybdic acid; above this, there is no increase in the net color intensity. The color stability is unaffected by changing molybdic acid concentration. When the ratio of the sodium molybdate to sulphuric acid in the molybdic acid solution was increased threefold, color production rose to a maximum between 0.5 and 1% molybdic acid. Above this, the color produced in all tubes, including the reagent blanks, rapidly increased but the net color intensity with a given phosphate standard decreased.

The sensitivity of the method to the stannous chloride reagent was studied by analyzing a given phosphate standard over a range of stannous chloride concentrations. The color intensity in the standards and corresponding reagent blanks increased slowly as the concentration was increased from 0.005 to 0.1% and then increased more rapidly. There was no increase in the net color intensity over this large range, however. These results may possibly explain the criticism of this method expressed by Roe and Kahn (9) who claimed that the values obtained varied with the amount of stannous chloride reagent added. Thus, it is true that the intensity of color produced with a given phosphate standard increases slightly as the concentration of the stannous chloride is increased, but when the corresponding reagent blank is set to 100, there is no change in the net color intensity. The color stability was not affected by changing stannous chloride concentration.

The effect of trichloroacetic acid on the intensity and stability of the color produced with a given phosphate standard (2.5 mgm. % P) was studied by varying the concentration of it used in Step 1. The results are shown in Fig. 3, the colorimeter having been set to 100 with distilled water in place. It can be seen that the stability of the color after the first 10 min. is unaffected by the amount of trichloroacetic acid present. The intensity of the color produced increases as the concentration of trichloroacetic acid is decreased. This suggests that the sensitivity of the method is also increased by decreasing the amount of trichloroacetic acid present. This was the conclusion reached

by Shinowara, Jones, and Reinhart (11) who recommended developing the color in a nearly neutral solution and reading in exactly six minutes since the color was unstable with time. A different conclusion is reached, however, when the

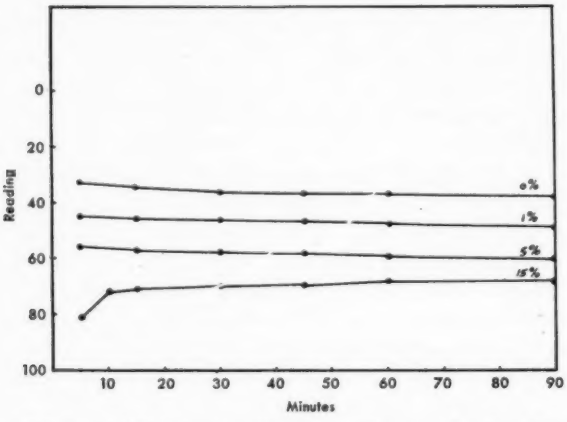

FIG. 3. *Effect of trichloroacetic acid concentration on color intensity and stability.*

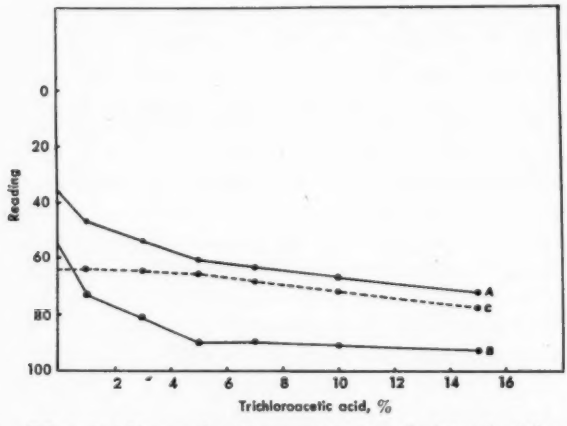

FIG. 4. *Effect of trichloroacetic acid concentration on absolute color (A), reagent blank (B), and net color (C).*

complete results in Fig. 4 are considered. If the curves shown in Fig. 4 are made only five minutes after the addition of the stannous chloride reagent, there is a small increase in the net color intensity as the trichloroacetic acid concentration is decreased but this is before the color has reached its level of maximum stability. The curves shown in Fig. 4 and described below were obtained after 15 min. and showed a drift of only two to three scale divisions up to two hours. Three curves are shown; *A* represents the values obtained

when the colorimeter is first set to 100 with distilled water in place, *B* is the color in the corresponding reagent blanks, and *C* is the curve obtained when the standards are read after the colorimeter has been set to 100 with the corresponding reagent blank in position. From Curve *A* it can be seen that the color intensity increases rapidly as the amount of trichloroacetic acid is reduced. This is the same effect shown in Fig. 3, apparently suggesting that the sensitivity of the method is also increased by decreasing the acidity. From Curve *B*, however, it is seen that the color in the reagent blanks is also greatly increased. Curve *C* shows that from 0 to 5% trichloroacetic acid, there is no increase in the net color intensity.

Thus, in the stannous chloride method, the net color intensity and stability are relatively independent of the molybdic acid concentration over the range 1 to 10% tested, independent of the stannous chloride concentration over the range 0.005 to 0.1% and independent of the trichloroacetic acid concentration over the range 0 to 5%. Under these conditions, this method is considerably more sensitive and no less stable than the method of Fiske and Subbarow.

Ammonium Vanadate Method

The reagents were freshly prepared as outlined by Simonsen *et al.* (12); 0.25% ammonium vanadate (Step 2), 5% ammonium molybdate (Step 3).

The effect of varying the concentration of the ammonium vanadate was studied in the usual manner. It was found that the intensity was unchanged over a concentration range of 0.1 to 1% and was completely stable over the two hour period tested. The color intensity, however, is inadequate for a micromethod under the present conditions.

The color intensity and stability were found to be independent of the amount of trichloroacetic acid over the range of 0 to 15% tested.

Increasing the concentration of ammonium molybdate over the range 1 to 20% caused an increase in the color of the phosphate standards and reagent blanks but did not increase the net color intensity.

The Fiske and Subbarow method using aminonaphtholsulphonic acid is widely used and is considered to have the advantage over the stannous chloride method of producing a color that is more stable although less intense. The use of stannous chloride, on the other hand, has the advantage of producing a greater color intensity and so an increased sensitivity; a disadvantage often cited is that the color is less stable and so the time factor must be carefully controlled.

This present investigation has shown that the ammonium vanadate method gives the greatest color stability but is not sufficiently sensitive for microbiological determinations. The Fiske and Subbarow method was found to be quite insensitive to the amounts of the various reagents present over a considerable range whose limits have been described quantitatively. The method can be considered to be stable, if by stable it is understood that the phosphate standards or unknowns give a fairly constant reading over a two hour period

of time when the reagent blank is taken into account before each reading. The method is less stable, however, than is generally considered when a calibration curve prepared, for example, after five minutes, as is usually the case, is used to read the standards or unknowns at a later time. That is, the color in both standards and reagent blanks does undergo some change with time, a value constant to within 0.3 mgm. % P only being obtained when the reagent blank is taken into account at each time.

The stannous chloride method is equally insensitive to the reagents present under the conditions defined when the reagent blank is taken into account before each reading. Under the conditions defined, it is more sensitive than the Fiske and Subbarow method and no less stable. The use of sodium molybdate in the stannous chloride method has the disadvantage that it is alkaline and on standing in a bottle, forms silicates from the glass. Silicate reacts similarly to phosphate in this reaction and, as the sodium molybdate ages, the color in the reagent blank increases. Ammonium molybdate does not have this disadvantage.

It was therefore proposed to devise a method replacing sodium molybdate with ammonium molybdate for greater reagent stability and to use stannous chloride for the greatest sensitivity. After a series of investigations on the different reagents such as were conducted above, the following method was developed.

Reagents

(1) Molybdic acid reagent. (*A*) Dissolve 2.5 gm. of reagent-grade ammonium molybdate in 97.5 ml. of distilled water. If there is any turbidity, the solution should be filtered. (*B*) Dissolve 150 gm. of reagent-grade trichloroacetic acid in 850 ml. of distilled water. Then add (*A*) to (*B*), mix well, and store in a dark bottle in the cold. This reagent is stable.

(2) Stannous chloride, stock solution. Dissolve 6 gm. of reagent-grade stannous chloride in 10 ml. of concentrated hydrochloric acid. Store in a dark bottle in the cold. This reagent is quite stable.

(3) Stannous chloride, dilute analytical reagent. Add 0.2 ml. of the stock stannous chloride to a 100 ml. volumetric flask and make up to the mark with distilled water. This solution is not stable for more than about six hours and so should be made up only for immediate use.

Procedure

(1) To a colorimeter tube add 10.0 ml. of the molybdic acid reagent.

(2) Add 0.5 ml. of the protein-free filtrate prepared as described on Page 1 (1).

(3) In about five minutes, add 0.5 ml. of the freshly prepared dilute stannous chloride reagent.

(4) Read in 15 min. or later in a photoelectric colorimeter using a 660 mμ filter. The instrument is set to 0 (to 100 with an Evelyn colorimeter) with a reagent blank in place. The reagent blank is prepared as above except that 0.5 ml. of distilled water is used in Step 2 instead of protein-free

filtrate. The phosphorus content of the samples is read from a calibration curve between colorimeter readings and phosphorus content established by treating known solutions of monopotassium phosphate over the range 2.5 to 10 mgm. % of phosphorus by this procedure. The phosphorus standards are run at the same time as the unknowns.

TABLE I

ANALYSIS AND RECOVERY OF PHOSPHATE IN PLASMA

Sample	Phosphorus found, mgm. %	Phosphorus added, mgm. %	Phosphorus recovered, mgm. %
1	4.4	2.50	2.60
	4.5	2.50	2.55
	4.5	2.50	2.50
	4.5	2.50	2.55
2	5.6	5.00	5.10
	5.5	5.00	5.00
	5.6	5.00	5.10
	5.6	5.00	5.00

In Table I are shown the results of a typical analysis on samples of plasma, each sample being analyzed in quadruplicate to show the degree of precision of the method. Also shown are the results of recovery experiments, obtained by adding known amounts of inorganic phosphate to the previously analyzed samples.

From the above analyses it can be seen that the method is accurate within $\pm 2\%$.

If only the inorganic phosphate level of plasma or serum is to be determined, a 0.1 ml. sample is used.

(1) Add 0.1 ml. of plasma or serum to 1.0 ml. of distilled water, rinsing the pipette by drawing the water up and down a few times.

(2) Add 1.0 ml. of 15% trichloroacetic acid, allow the mixture to stand for about 15 min. and then centrifuge.

(3) The analysis is performed as outlined in the procedure above except that 1.0 ml. of this protein-free supernatant is used in Step 2. The calibration curve is prepared by treating phosphorus standards in exactly the same manner as the plasma or serum.

If necessary, the analysis can be performed on a 0.02 ml. sample of plasma or serum as follows:

(1) Add 0.02 ml. of plasma or serum to 1.5 ml. of distilled water.

(2) Add 1.0 ml. of 15% trichloroacetic acid, allow the mixture to stand for about 15 min., and then centrifuge.

(3) Add 2 ml. of this protein-free supernatant to 5.0 ml. of the molybdic acid reagent.

(4) In about five minutes, add 0.5 ml. of the freshly prepared dilute stannous chloride reagent. The calibration curve is prepared by treating phosphorus standards in exactly the same manner as the plasma or serum.

The inorganic phosphate in urine can be determined by first diluting it 1 : 50 in 0.1% trichloroacetic acid. A 0.1 ml. sample is then treated in the same manner as 0.1 ml. of plasma or serum.

This method is simple and rapid and is accurate to within $\pm 2\%$. It is sufficiently sensitive to give quantitative color development with less than 1γ of phosphorus. The color produced is quite stable for colorimetry; a calibration curve between colorimeter readings and standards of known phosphorus content prepared 15 min. after the addition of the stannous chloride reagent can be used to measure the phosphorus content of unknowns over a period of one hour with an error of less than 0.2 mgm. % P.

Acknowledgment

The author wishes to take this opportunity to thank Dr. J. B. Collip and Dr. J. C. Paterson for their encouragement of this work.

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THE EFFECT OF IODOACETATE ON THE APPARENT SYNTHESIS OF DIPHOSPHOTHIAMINE DURING THE ESTIMATION OF THE COCARBOXYLASE ACTIVITY OF BLOOD EXTRACT[1]

By M. C. Blanchaer and L. H. Cohen

Abstract

The usual method of determining diphosphothiamine (DPT) by its cocarboxylase activity proved unreliable when applied to blood extract because of a marked upward drift of the results during the period of measurement. This phenomenon, which was associated with excessively high recovery values, was tentatively attributed to a synthesis of DPT since the presence of 1×10^{-3} molar iodoacetate abolished the increase in carboxylase activity. Judging from recovery experiments, the addition of iodoacetate to the reactants permitted a more reliable estimation of the DPT content of blood extract.

Introduction

Diphosphothiamine (DPT) in blood and tissues is commonly estimated by its cocarboxylase activity in the decarboxylation of pyruvate by yeast.

$$CH_3COCOOH \xrightarrow[\text{Yeast carboxylase}]{\text{DPT}} CH_3CHO + CO_2$$

Addition of tissue extracts to this system is reported to result in the synthesis of small amounts of DPT from thiamine and phosphate (2). However, the quantities formed are apparently insufficient to interfere with the determination of DPT in muscle, liver, kidney, and brain (3, 4). The present report deals with a similar effect occurring in the presence of blood extract and with its influence on the estimation of DPT in blood.

Methods

1. *Preparation of Blood Extract*

Blood extract was prepared as described by Westenbrink *et al.* (7). Two ml. of fresh defibrinated human blood was added to an equal volume of 0.28 *N* hydrochloric acid and heated with continuous stirring for one and one-quarter minutes in a boiling water bath. Upon removal from the bath 0.5 ml. of 6.5% potassium hydroxide was added with vigorous stirring. The preparation was immediately centrifuged for 15 min. at 2500 r.p.m. in order to clear the supernatant blood extract. In recovery experiments, 0.25 volumes of a 10 or 20 μgm. % DPT solution was added to one volume of the extract. An equal volume of water replaced the DPT solution in the corresponding control estimations.

[1] *Manuscript received June 15, 1949.*

Contribution from the Nutrition Laboratory, Department of Physiology and Medical Research, University of Manitoba, Winnipeg, Man.

2. *Preparation of Yeast Carboxylase*

Dried brewer's yeast was washed with alkaline phosphate buffer just before use to remove preformed DPT. The procedure was as follows: 0.6 gm. of powdered yeast was suspended, with stirring, in 50 ml. of 0.1 *M* phosphate buffer, pH 9.0, and the supernatant was discarded after centrifuging. This process was repeated with the same buffer, again with water, and finally with 0.1 *M* phosphate buffer pH 6.2. Exactly four minutes was allowed for suspension of the yeast in the various solutions and three minutes for each centrifugation at 2500 r.p.m.

The washed yeast was resuspended for use in 6.0 ml. of 0.1 *M* phosphate buffer, pH 6.2, containing 1.0 mgm. each of magnesium and manganese ions as the chloride salts and 1.0 mgm. of thiamine chloride. Thiamine, magnesium, and manganese serve as activators of yeast carboxylase (3).

Since calcium interferes with the estimation (6), all water used in washing the yeast and the preparation of reagents was redistilled in glass over potassium carbonate. The distillate was discarded until neutral to methyl red.

3. *Determination of Cocarboxylase Activity*

The cocarboxylase activity of blood extract was estimated by its ability to stimulate the decarboxylation of pyruvate (7). Carbon dioxide produced in the reaction was measured manometrically (5) in 15 ml. Warburg flasks at 27.5° C. in an atmosphere of air. The activity of standard amounts of DPT (0.00, 0.05, 0.10, and 0.20 μgm. in 2 ml. of water) was determined simultaneously.

The following were placed in the main compartment of the Warburg flasks: 0.2 ml. of 2 molal phosphate buffer, pH 6.2; 2.0 ml. of blood extract or standard DPT solution; and 0.5 ml. of the final yeast suspension. In certain experiments indicated below, sufficient sodium iodoacetate was dissolved in the 2 molal buffer to bring the contents of the flasks to a final iodoacetate concentration of 1×10^{-3} molar. The side arm of the flasks contained 5 mgm. of sodium pyruvate in 0.1 ml. of 0.1 *M* phosphate buffer pH 6.2.

The flasks, attached to manometers, were equilibrated for 10 min. in the bath. The pyruvate was then tipped in and the shaking continued. At the end of five minutes, zero readings were taken and the taps closed. Carbon dioxide production was measured at varying intervals for periods up to 70 min. At each time interval the apparent DPT content of the blood extract was read directly from the corresponding standard curve (Fig. 1). In analyzing the results, variation from the mean was expressed as the standard deviation.

Results

In the flasks containing DPT dissolved in water, the rate of carbon dioxide production remained constant for approximately 50 to 60 min. following a short initial lag period. The total volume of gas released at any given time was usually proportional to the DPT content of the flasks over the range

0.00 to 0.20 μgm. DPT (Fig. 1). In the vessels containing blood extract, decarboxylation followed a similar course for a short time and then showed

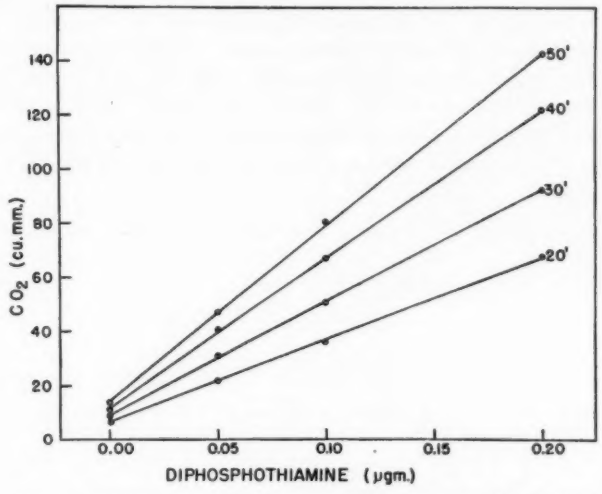

FIG. 1. *Typical standard curves of carbon dioxide production at 20, 30, 40, and 50 min.*

a gradual acceleration that became more marked as time elapsed (Fig. 2A). Table I shows the effect of this phenomenon on the apparent DPT content of an extract prepared from another sample of blood. The upward trend of

TABLE I

APPARENT DPT CONTENT OF BLOOD EXTRACT (μgm.) AT VARIOUS TIMES

Time (min.)	Blood extract			Blood extract + 0.04 μgm. DPT			Mean recovery* (%)
	Flask 1	Flask 2	Mean (1)	Flask 3	Flask 4	Mean (2)	
10	0.078	0.069	0.074	0.100	0.126	0.113	98
15	0.077	0.068	0.073	0.113	0.137	0.125	130
25	0.087	0.081	0.084	0.144	0.148	0.146	155
30	0.090	0.087	0.088	0.161	0.177	0.169	202
35	0.097	0.097	0.097	0.177	0.196	0.187	225

* $Recovery\ (\%) = \frac{mean\ (2) - mean\ (1)}{0.04} \times 100.$

the results is representative of similar changes in 18 additional experiments in which the average values increased 15% ± 7 between the 20 and 40 min. readings. This drift was accentuated by the addition of DPT to the extract as shown in Table I. In 16 additional recovery experiments cocarboxylase activity increased 24% ± 8 between the 20 and 40 min. readings, resulting in recovery values that rose from 119% ± 19 to 159% ± 20.

Since the method as described was obviously unreliable, it was felt that a study of the more probable explanations for the upward drift might form a basis for improving the procedure. Goodhart and Sinclair (1) reported a similar increase in the values, which they ascribed to a decrease in carbon dioxide production in the standard flasks due to a drop in pH after the first 30 min. In the present study, adequate buffering maintained the initial pH of 6.2 for at least one hour. The nature of the gas production curves (Fig. 2A) also indicated that the effect was due to an increased activity in the presence of blood extract and not to a decreased rate of decarboxylation in the standard flasks.

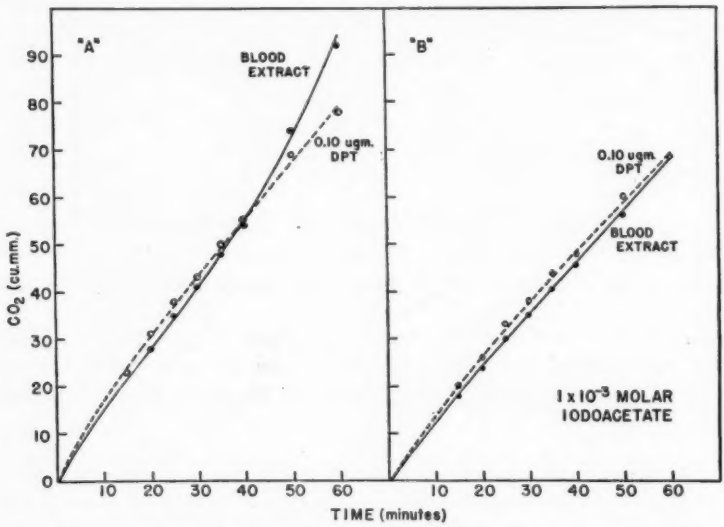

FIG. 2. *A comparison of the rate of carbon dioxide production in the presence of blood extract and 0.1 μgm. DPT, with and without iodoacetate.*

The possibility of DPT being synthesized during the course of the estimation was next examined. Unfortunately, a direct investigation of changes in the DPT content of the extract during the decarboxylation reaction was impossible because of the lack of reliable independent methods for estimating this substance. However, indirect evidence was obtained by adding to the system iodoacetate, which is stated to inhibit the synthesis of DPT (2). The results shown in Fig. 2A and B were obtained simultaneously using the same blood extract. It will be seen that decarboxylation in the presence of 1×10^{-3} molar iodoacetate was slightly depressed. However, gas production in the vessel containing blood extract followed an almost linear course similar to that in the standard flask. The inhibitory effect of iodoacetate on the drift of the values with time is evident when the results in Table II are compared with those in Table I. In eight additional determinations using iodoacetate

TABLE II

APPARENT DPT CONTENT OF BLOOD EXTRACT (μgm.) AT VARIOUS TIMES IN THE PRESENCE OF 1×10^{-3} MOLAR IODOACETATE

Time (min.)	Blood extract			Blood extract + 0.08 μgm. DPT			Mean recovery* (%)
	Flask 1	Flask 2	Mean (1)	Flask 3	Flask 4	Mean (2)	
20	0.070	0.080	0.075	0.153	0.166	0.160	106
25	0.070	0.079	0.075	0.157	0.166	0.162	107
30	0.067	0.076	0.071	0.153	0.161	0.157	107
40	0.067	0.073	0.070	0.157	0.160	0.158	110
50	0.068	0.072	0.070	0.153	0.160	0.157	109
60	0.068	0.077	0.072	0.152	0.157	0.155	102
70	0.063	0.075	0.069	0.151	0.156	0.154	106

* $Recovery\ (\%) = \frac{mean\ (2) - mean\ (1)}{0.08} \times 100.$

the 40 min. values decreased 1% ± 5.3 from the 20 min. readings. Over the same period, the mean recovery of DPT in seven experiments increased from 105% ± 7.4 to 107% ± 10.4.

Discussion

The results reported here show that iodoacetate abolishes the increase in carboxylase activity that occurs with time. Judging from the recovery experiments, the use of iodoacetate permits a more reliable estimation of the DPT content of blood extract. Further work is required to establish whether the rise in carboxylase activity inhibited by iodoacetate truly represents a synthesis of DPT.

Since workers (1, 7) using previous methods for estimating DPT in blood have failed to describe an upward drift of their results, it seems possible that our difficulties arose from the use of a strain of yeast with an unusually great ability to synthesize DPT. For this reason, the results presented above do not necessarily invalidate previously published methods for estimating DPT in blood. Nevertheless, the usual practice of terminating such determinations 30 min. after the addition of pyruvate limits the potential sensitivity and accuracy of the estimations because of the large percentage error involved in measuring the small volumes of gas produced during this short initial period.

The present study indicates that the presence of iodoacetate in appropriate concentrations largely overcomes the objections to the prolonging of the determinations for an additional 30 min., thereby permitting an increasing degree of sensitivity and accuracy in DPT estimations on blood extract.

Acknowledgments

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LIPOTROPIC ACTIVITY OF CHOLINE, BETAINE, AND METHIONINE IN DUCKLINGS[1]

By R. Bernard and J. M. Demers

Abstract

A purified diet, deficient in choline and containing 18% casein, results in fatty infiltration of the liver in ducklings killed at four weeks. Total liver lipids is about 13%, the normal value being 6 to 7%. Addition of 0.1 or 0.3% choline chloride results in nearly normal livers. In this respect, ducklings differ from chicks and are comparable to rats and dogs. Betaine has a slight lipotropic activity but does not stimulate growth and has no antiperotic properties. DL-methionine, when added to a purified diet containing 9% casein or to a simplified diet containing 15% casein, has no lipotropic activity. On the contrary, this amino acid increases the total lipids and the severity of fatty infiltration of the livers. Choline has a triple function in ducklings; it promotes growth, prevents hepatic fatty infiltration, and perosis.

Introduction

It is now well established that choline controls fat deposition in the liver of mammals such as rats, dogs, and pigs (5, 4, 8). In rats fed choline deficient diets, the livers are enlarged and pale in color. Microscopically, fat droplets are seen in the hepatic cells. The droplets are frequently large enough to push the cytoplasm and nucleus aside. Moreover, Griffith and Wade (10) found that, if young rats are used, a severe renal hemorrhagic degeneration occurs. The addition of 0.1% choline to a deficient diet prevents fatty livers and kidney damage in rats. In this animal, betaine is also effective and its activity has been found to be about one-third that of choline (25). Methionine also protects young rats against hemorrhagic degeneration of the kidney (11) and its lipotropic activity is calculated as one-fifth that of choline (7). The feeding of a diet containing 30% casein supplies enough methionine to obviate the necessity of a choline supplement (24).

Among birds, the effect of choline in promoting growth and preventing perosis has been studied in chicks and young turkeys (15, 17) as well as in ducklings (26, 3). Perosis is characterized by a shortening and thickening of the tarsus and tibia of young birds. Usually a distortion and dislocation of the hock joint results in the slipping of the tendo calcaneus.

Chicks and turkey poults, when fed purified diets, appear to be unable to utilize betaine or methionine as substitutes for choline. However, when simplified diets that contain peanut meal and yellow cornmeal are fed, these supplements promote growth and have antiperotic properties (14, 16, 21, 22).

In rats, methionine and betaine are effective substitutes for choline. Both substances form choline in the body by methylation of ethanolamine as shown by du Vigneaud and Stetten (30, 31). According to Jukes *et al.* (18) such a

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reaction does not occur in the chick. Almquist (2) has set forth the contrast between mammalian and avian nutrition in respect to interrelations between choline, betaine, and methionine.

The lipotropic activity of choline in bird nutrition has received the attention of a few workers. Kilborn (19) found that adult ducks and pigeons fail to show increased liver fat on a choline-free, high-fat diet and in this way they differ radically from mammals (rats and dogs). Shen (27) has shown that force feeding in ducks produces a marked increase in liver fat, and Flock *et al.* (9) have shown the same to be true of geese. Abbott and De Masters (1), on the basis of analyses of seven samples only, claim that choline decreases the percentage of fatty acids in the liver of chickens killed when 290 days old. Hegsted *et al.* (13) found that choline has no lipotropic action on the livers of chicks killed when four weeks old. It thus appeared from these results that the main functions of choline in avian nutrition are limited to the prevention of perosis and the stimulation of growth.

In previous work on the nutrition of ducklings, the authors (3) noticed that choline deficient birds presented large yellow livers apparently rich in fat. Frozen sections of the livers of the deficient birds revealed intense fatty infiltration and it was decided to study at length the lipotropic activity of choline, betaine, and methionine in this avian species.

Material and Methods

Day-old white Pekin ducklings were used in these studies. Upon receipt from the hatchery, they were placed in heated batteries equipped with raised-screen bottoms. Six ducklings were located in each compartment, measuring about 35 in. square. The birds used in experiments involving purified diets were fed a commercial duck feed for two days prior to the experimental period. Food and water were allowed ad libitum. Each group consisted of six ducklings and the experimental periods lasted three or four weeks. The birds were then killed and samples of liver were obtained for chemical and histological analyses. Liver fat (total lipids) was determined on fresh samples according to the procedure of Shipley *et al.* (28). Pieces of liver fixed in 10% formalin were sectioned with the freezing microtome and stained with Sudan IV and hematein.

The composition of the basal diets used is given in Table I. Diet R-49 is patterned after that used by Hegsted (12) in his studies on duck nutrition. The low-casein diet R-53 is similar to those generally used to produce methionine deficiency in the rat. The simplified (semipurified) diet R-2 resembles that used by Jukes (14) in his early work on perosis. The three basal diets differ principally by their casein contents but resemble each other in being deficient in choline.

Three series of experiments were carried out. In the first series, the lipotropic activity of choline and betaine was investigated, using diet R-49, which contains 18% casein. The second series involved the study of the

TABLE I

COMPOSITION OF BASAL DIETS USED IN THE STUDY OF THE LIPOTROPIC ACTIVITY OF CHOLINE BETAINE, AND METHIONINE IN DUCKLINGS

Ingredients	Basal diet number		
	R-49	R-53	R-2
	Composition		
	%		
Main ingredients			
Sucrose	47		
Cornstarch		70	
Corn meal, yellow			65
Casein, Labco	18	9	10
Skim milk, dry			15
Gelatin	10		
Corn oil	10	10	
Soybean oil			3
Fish oil blend*	2	2	1
Alfalfa meal			1
Salts IV**	5	5	5
$CaH_4(PO_4)_2 2H_2O$	1		
Liver fraction L†	4		
Ruffex	3	3	
	Mgm. per 100 gm. of diet		
Vitamins			
Thiamine hydrochloride	0.4	0.4	0.4
Riboflavin	0.8	0.8	0.8
Calcium pantothenate	2.5	2.5	1.5
Nicotinic acid	4.0	4.0	3.0
Pyridoxine hydrochloride	0.4	0.4	0.4
p-Aminobenzoic acid	2.0	2.0	
Biotin	0.02	0.02	
Inositol	100.0	100.0	
2-Methyl naphthoquinone	0.1	0.1	0.1
α-Tocopherol	10.0	10.0	10.0
Pteroylglutamic acid		0.1	

* *Vadol Type No. IV, 3000 A, 400 D (A.O.A.C. chick units). Blended fish oils fortified with vitamin* D_3.

** *Hegsted, D. M., Mills R. C., Elvehjem, C. A., and Hart, E. B. J. Biol. Chem. 138 : 459. 1941.*

† *A source of the unknown vitamins of the B-complex. Supplied by Wilson Laboratories, Chicago, Ill.*

lipotropic activity of methionine and choline with the low-methionine diet R-53 containing only 9% casein. Finally, the lipotropic activity of methionine and choline was studied in ducklings fed a semipurified diet containing approximately 15% casein, which is intermediate between the two levels mentioned above.

Results and Discussion

A. Effect of Choline and Betaine Using Basal Diet R-49, Containing 18% Casein

The results of this first series of experiments are presented in Fig. 1 and Table II. The growth curves show that at four weeks of age, ducklings fed

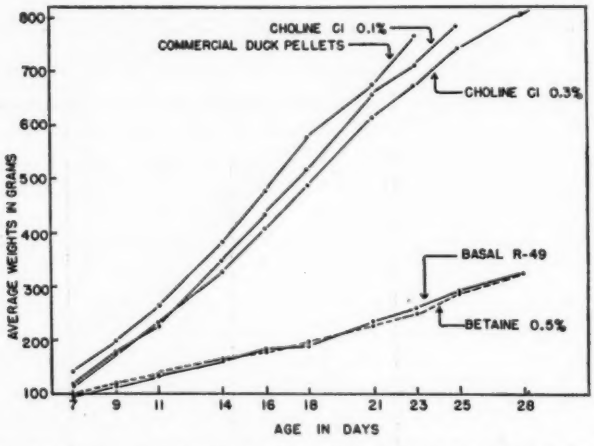

FIG. 1. *Growth curves of ducklings fed purified choline deficient basal diet R-49 (18% casein) only or the same diet supplemented with choline or betaine as compared with commercial duck pellets.*

TABLE II

EFFECT OF SOME LIPOTROPIC FACTORS WHEN ADDED TO AN 18% CASEIN, PURIFIED DIET

Group No.*	Supplement to basal diet R-49	Number with perosis	F.I.**	Liver lipids, %	t††
81	None	6	5	14.4 ± 1.46†	
82	Choline chloride 0.1%	1	2	8.0 ± 1.29	3.26
83	Choline chloride 0.3%	0	3	8.0 ± 0.36	4.27
84	Betaine 0.5%	6	6	10.8 ± 1.95	1.77
80	Commercial duck pellets only	0	0	6.0	

* *Six birds in each group.*

** *Number with fatty infiltration.*

† *Standard error* $= \sqrt{\left(\frac{\Sigma d^2}{n(n-1)}\right)}$.

†† $t > 2.30$ *significant by comparison with control group.*

basal diet R-49 weighed about one-third as much as normal individuals fed a commercial duck feed containing more than 24% protein. The supplementation with 0.5% betaine has no effect on growth, while choline at levels of 0.1 or 0.3% resulted in a nearly normal growth. In Table II are found the observations on perosis, fatty infiltration, and total lipids. All ducklings fed the

basal diet showed clear-cut signs of perosis. At a level of 0.3%, choline prevented the malformation entirely. Since one perotic individual was noted in the group supplemented at the 0.1% level, it appears that this particular level is too low for complete protection against perosis but may be satisfactory for optimum growth.

The average lipid content of livers of ducklings fed the commercial diet was 6% and no stainable fat could be detected. On the other hand, the livers of all birds fed the choline deficient diet showed fatty infiltration (Fig. 5*a*) and averaged 14.4% total lipids. The livers of the animals in this last group were pale yellow, soft, and some of them were enlarged. The addition of 0.1 or 0.3% choline chloride resulted in a significant reduction in the liver lipids and the fatty infiltration. However, the percentage of total lipids was not brought entirely back to normal and a few individuals showed some fatty infiltration though it was of a very slight degree. This may indicate that a diet containing 18% casein supplemented with 0.3% choline chloride is still deficient in lipotropic factors. Beveridge *et al.* (6), working with rats, suspect that in addition to methionine, tyrosine may have some lipotropic properties.

These results indicate that in ducklings, choline fulfils three functions: it promotes growth, prevents perosis, and has a definite lipotropic activity. This last function has not been observed in the chick.

Betaine at the level of 0.5% does not improve growth and has no antiperotic properties but rather appears to enhance the symptoms of perosis. There is some indication that this compound has lipotropic activity though the results are not statistically significant. The livers of this group of ducklings were normal in size and color, i.e., dark brown. Microscopic examination revealed a small amount of diffuse fat as compared with the well defined droplets observed in livers of the basal group. It is probable that betaine may favor the synthesis of a limited amount of a cholinelike substance that is used up only for fat transportation and is ineffective in preventing perosis or promoting growth. The results with betaine also suggest that in ducklings as in chicks, the choline requirement may be divided into two parts which McKittrick (23) calls the essential choline and replaceable choline. In ducks, the lipotropic function seems to be the replaceable one. More work on this question is, however, necessary.

B. Effect of Choline and Methionine Using a Purified Diet, R-53, Containing 9% Casein

The results of the second series of experiments are presented in Table III. In all cases the growth was very poor, being on the average about one-fifth that of normal, even when both supplements were added to the basal diet. This is no doubt due to the fact, as was suggested by Knowlton Hall and Sydenstricker (20), that such a diet is known to be suboptimal for many essential amino acids. No signs of perosis were observed, even in the basal

TABLE III

EFFECT OF SOME LIPOTROPIC FACTORS WHEN ADDED TO A 9% CASEIN, PURIFIED DIET

Group No.*	Supplement to basal diet R-53	F.I.**	Liver lipids, %	t	Average weights, gm.	
					4 days	21 days
85†	None	6	34.3 ± 7.48		79	146
86†	DL-methionine 0.5%	6	47.7 ± 2.39	1.70	76	192
87	None	6	29.7 ± 7.06		94	175
88	DL-methionine 0.5%	6	29.8 ± 5.12		86	132
89	Choline chloride 0.3%	4	17.5 ± 5.22	1.39	78	122
90	DL-methionine 0.5%, choline chloride 0.3%	6	14.8 ± 2.69	1.98	70	151

* *Six birds in each group.*
** *Number with fatty infiltration.*
† *Groups 85 and 86 started one month earlier.*

groups. This is probably related to the poor growth rate as has been shown in chicks.

The lipotropic activity of methionine was first investigated by comparing Groups 85 and 86. Much to our surprise, the addition of this amino acid appeared to increase the total lipids of the livers, although the difference is not quite significant. These findings could not be duplicated for Groups 87 and 88, which showed the same average percentage in liver lipids. The livers of ducks fed basal diet R-53 alone or with added methionine were large, pale yellow, or creamy white. Results with Groups 86 and 88 show that, in ducklings, methionine is unable to replace choline as a lipotropic factor. Added evidence was obtained on the lipotropic activity of choline in Groups 89 and 90. In this last group, it is believed that methionine when added with choline may help in decreasing the liver lipids.

In this series of experiments with the low-casein diet, the fatty metamorphosis of livers was most marked in groups in which the diets were supplemented with methionine (Fig. 5*b*). Great individual variations in liver lipids was noted in all groups and this is perhaps related to a multiple deficiency in essential amino acids.

C. Effect of Choline and Methionine Using a Simplified Basal Diet, R-2, Low in Choline and Containing Approximately 15% Casein

In order to gain more information on the importance of methionine, a third series of experiments was conducted using a simplified basal diet (R-2) containing approximately 15% casein. It was thus hoped to avoid multiple amino acids deficiencies and yet still have a low-methionine diet.

The growth curves corresponding to the treatments studied in this series are shown in Fig. 2. The addition of methionine to the basal diet R-2 did not improve growth. On the contrary, there seems to be a slight inhibition. This is supported by the fact that when methionine and choline are added together, the growth is inferior to that when choline alone is added. Perosis

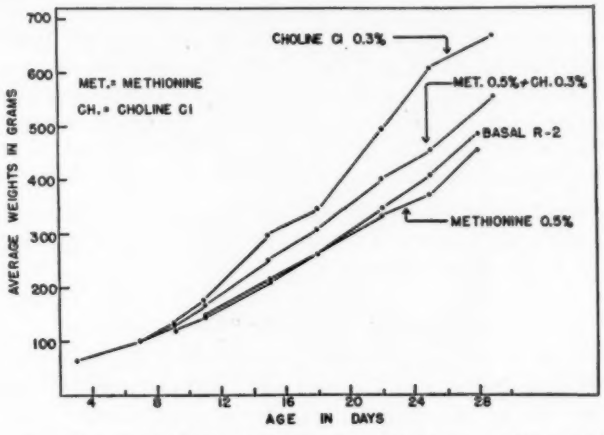

FIG. 2. *Growth curves of ducklings fed simplified choline-low basal diet R-2 (15% casein) only or the same diet supplemented with choline or methionine or both.*

TABLE IV

EFFECT OF SOME LIPOTROPIC FACTORS WHEN ADDED TO A 15% CASEIN, SEMIPURIFIED DIET

Group No.*	Supplement to basal diet R-2	Number with perosis	F. I.**	Liver lipids, %	*t*
91	None	3	6	16.7 ± 1.37	
92	Choline chloride 0.3%	0	6	13.2 ± 2.11	1.41
93	DL-methionine 0.5%	3	6	21.8 ± 2.64	1.70
94	DL-methionine 0.5%, choline chloride, 0.3%	0	6	10.4 ± 1.50	3.12

* *Six birds in each group.*
** *Number with fatty infiltration.*

was observed in the basal group (Figs. 3 and 4) and in that supplemented with methionine, while the two groups receiving 0.3% choline chloride, with or without added methionine, were entirely protected. It thus appears that methionine is unable to replace choline for this function in this simplified diet.

The livers of ducklings fed the basal diet R-2 contained on the average 16.7% fat. Choline showed a very slight lipotropic effect, while methionine increased the liver lipids to 21.7%. This confirms the results obtained with

the 9% casein diet, in that methionine in the experimental conditions outlined is antilipotropic. When both supplements are added (Group 94), there is a significant decrease in liver lipids to 10%, although this is still 4% above normal.

Fatty infiltration was noticed in all the birds of this series of experiments. It was most pronounced in Group 93, supplemented with methionine, and the least in Group 94, which received both supplements. Choline alone had a definite lipotropic action as shown by the comparison of Figs. 5*c* and 5*d*. It is evident that the basal diet R-2 containing 15% casein even when supplemented with methionine and choline is still deficient in lipotropic factors and this probably can be corrected by a higher percentage of casein. Looking back to the first series of experiments, it will be seen that, when an 18% casein basal diet is used, choline alone brought the liver lipids practically to normal.

From the results presented in this work, it is suggested that there exists a marked difference between chick and duckling in so far as fat accumulation and transport in the liver is concerned.

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PLATE I

FIG. 3. *Perotic duck. Age: 16 days. Diet R-2 low in choline. Note toes curled in.*

FIG. 4. *Same animal as above. The flattening of the tibiometatarsal joints and deviation of the right leg are well shown.*

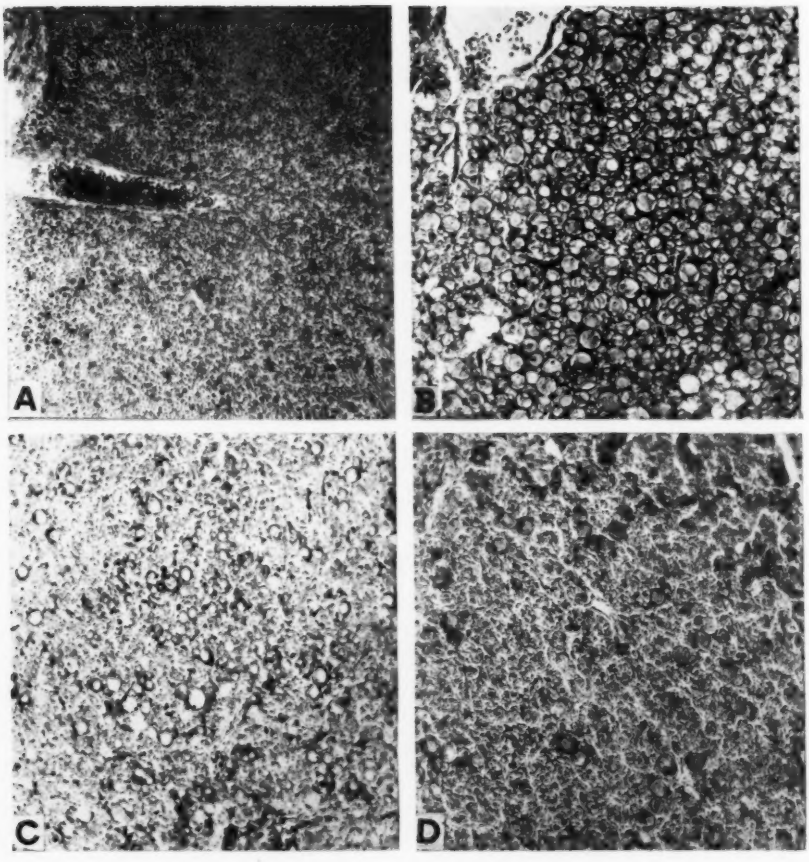

FIG. 5. *Photomicrographs of liver sections of ducklings. Frozen section stained with Sudan IV and hematein. Approx. × 113.*

A. Purified basal diet R-49 (18% casein), choline deficient. The fat droplets are small and uniformly distributed.

B. Purified basal diet R-53, choline-free and low in casein (9%) supplemented with 0.5% methionine. Note the large amount of fat infiltration.

C. Simplified diet R-2 (15% casein) low in choline. Fatty infiltration is extensive.

D. Diet R-2 plus 0.3% choline chloride. The fatty infiltration is reduced but still evident.

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PHOSPHATASE OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES[1]

By D. M. Cram and R. J. Rossiter

Abstract

Rabbit polymorphonuclear leucocytes contain an active phosphatase that readily hydrolyzes disodium phenyl phosphate. The pH activity curve of the enzyme was found to have two maxima, one in the region of pH 10 and the other in the region of pH 5. The alkaline phosphatase was much more active than the acid phosphatase. The concentration of alkaline phosphatase in rabbit white cells was approximately one thousand times that of the enzyme in the serum. Under the conditions of study, the alkaline phosphatase activity was proportional to the concentration of the enzyme. The effect of substrate concentration on the enzyme activity was studied and the Michaelis constant (K_s) determined. An excess of substrate inhibited the enzyme. The course of the reaction was linear with time for the first 60 min.; after 90 min. the activity fell off faster than would be expected if the reaction were of the first order.

Magnesium and glycine, in low concentrations, caused an increase in the enzyme activity, whereas zinc, cyanide, borate, phosphate, bile salts, and glycine, in higher concentrations, were inhibitory. Fluoride had no demonstrable effect. Surface-active substances, such as saponin, bile salts, or alkyl sulphate, liberated the enzyme from the cells. Similar results were obtained when α-glycerophosphate or β-glycerophosphate was used as the substrate.

The alkaline phosphatase can be considered to belong to Class AI of Folley and Kay (22) and the acid phosphatase to Class AII. The alkaline phosphatase can also be considered to be a Phosphatase II of Cloetens (9).

Introduction

Phosphatases, or perhaps more correctly, phosphomonoesterases, have been found in a great many tissues. References to most of the work on these enzymes are given in the excellent reviews of Kay (39), Folley and Kay (22), and Moog (47).

Kay (37, 38) suggested that white cells may contain a phosphatase and, subsequently, Roche (53, 54) demonstrated alkaline phosphatase activity in mixed white cell preparations containing both granulocytes and lymphocytes, obtained from the blood of the rabbit, guinea pig, or horse. Umeno (60) measured the phosphatase activity of white cells from the peritoneal exudate of a rabbit; no mention was made of the type of cell present or of the pH of the reaction mixture. More convincing, however, were the experiments of Fiessinger and Boyer (16) on the alkaline phosphatase of rabbit white cells. Both alkaline and acid phosphatase have also been demonstrated in bone marrow (27, 65).

Although these reports indicate beyond doubt that there is an alkaline phosphatase in white cells, they are quantitatively misleading. It has recently been shown (Rossiter (56)) that surface-active substances such as saponin, bile salts, or alkyl sulphate liberate large quantities of alkaline phosphatase from rabbit polymorphonuclear leucocytes into the surrounding

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Contribution from the Department of Biochemistry, University of Western Ontario. The research was supported by a grant from the National Research Council of Canada.

fluid. This phosphatase would not be measured if, as in previous experiments on white cell phosphatase, estimations were done on suspensions of intact cells. The difficulties of extracting enzymes from cells are very real, and, for phosphatases, the problem has been adequately discussed (22, 39). By using saponin to extract the enzyme from the leucocytes, some of the difficulties have been overcome. A preliminary account of these experiments has already appeared (Cram and Rossiter (11)).

Methods

Cell Preparations

Polymorphonuclear leucocytes were obtained from the rabbit by the method of de Haan (28). Warm sterile isotonic saline (300 to 500 ml.) was run into the peritoneal cavity and drained off three to four hours later into a beaker containing a few grains of heparin. Alternatively, the saline was left in the animal over night. A further quantity of saline was run in the following morning and withdrawn immediately. In each instance the resulting opalescent fluid contained 2000 to 16,000 leucocytes per cu. mm., at least 95% of which were polymorphonuclear.

Phosphatase Determination

Phosphatase was usually determined by the method of King and Armstrong (41), in which the phenol, liberated by the hydrolysis of disodium phenyl phosphate, is measured by the method of Folin and Ciocalteu (18). To a centrifuge tube was added:

2 ml. buffer (either acetate buffer pH 4.9 or carbonate–bicarbonate buffer pH 9.9),

2 ml. 0.01 *M* disodium phenyl phosphate,

0.5 ml. 1% saponin,

0.3 ml. 0.017 *M* magnesium chloride.

The tube was then placed in a 37° C. water bath and, after temperature equilibration, 0.2 ml. of the cell suspension was added. The cells of 0.2 ml. suspension were thus extracted by fluid containing 5 mgm. saponin or the cells of 1 ml. by fluid containing 25 mgm. saponin. This amount of saponin has previously been shown to be optimal (56). Exactly one hour after the addition of the cell suspension, 1.8 ml. Folin and Ciocalteu reagent (diluted 1 : 3) was added and, after mixing, the tube was centrifuged. The phenol in 4 ml. of the supernatant was then determined as described by King and Armstrong (41). The test was always run in duplicate. Control tubes were also set up in which all the substances of the test, except the cell suspension, were incubated for one hour. The Folin and Ciocalteu reagent was then added, followed by the cell suspension. The color of the experimental tube was read against that of the control tube in a Coleman Universal Spectrophotometer at 650 mμ.

In experiments with either α-glycerophosphate or β-glycerophosphate as substrate the inorganic phosphorus liberated by the enzyme was measured by

the method of Fiske and Subbarow (17) after the proteins had been precipitated with 3 ml. 20% trichloroacetic acid. The color was read in a Coleman Universal Spectrophotometer at 650 mμ.

Recording of Results

Usually the results were expressed as the amount of phenol or phosphorus (in mgm.) liberated by 100 ml. enzyme preparation in one hour. In the section on the quantitative determination of the enzyme in the leucocytes, the results were given in King–Armstrong (41) units, as subsequently modified (43), per 10^{10} cells. These results were also brought into line with other estimates of the concentration of enzymes in white cells by converting them into King–Armstrong units per mgm. acid-insoluble phosphorus, using the relation that 10^{10} cells contain 6.8 mgm. acid-insoluble phosphorus previously established for the rabbit polymorphonuclear leucocyte (Rossiter (57)).

Results

Concentration of Phosphatase in Rabbit Polymorphonuclear Leucocytes

The concentration of alkaline (pH 9.9) and acid (pH 4.9) phosphatase in rabbit polymorphonuclear leucocytes is given in Table I. The mean concentration of alkaline phosphatase was 207 (±68) King–Armstrong units

TABLE I

PHOSPHATASE ACTIVITY OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 *M*. INCUBATION TIME, 1 HR. TEMPERATURE, 37° C.

Cell count W.B.C./ cu. mm.	Alkaline phosphatase activity			Acid phosphatase activity		
	Mgm. phenol/ 100 ml. enzyme/hr.	King–Armstrong units/ 10^{10} cells	King–Armstrong units/mgm. acid-insol. phosphorus	Mgm. phenol/ 100 ml. enzyme/hr.	King–Armstrong units/ 10^{10} cells	King–Armstrong units/mgm. acid-insol. phosphorus
3000	42	343	50	1.2	10	1.5
3300	33	245	36	0.9	7	1.0
4100	30	189	28	2.1	13	1.9
7400	36	121	18	2.7	10	1.5
4000	18	113	17	0.9	1	0.2
6900	60	209	31	1.1	4	0.6
4200	36	216	32	1.0	6	0.9
1800	15	216	32	0.8	11	1.6
	Mean (±S.d.)	207 (± 68)	31 (± 10)	Mean (±S.d.)	7.8 (± 3.7)	1.1 (± 0.5)

per 10^{10} cells or 31 (±10) King–Armstrong units per mgm. acid-insoluble phosphorus. This corresponds approximately to 52 King–Armstrong units per gm. fresh tissue or 320 King–Armstrong units per gm. dry tissue. The concentration of acid phosphatase was much less (7.8 ± 3.7 King–Armstrong units per 10^{10} cells).

Other Substrates

In the experiments reported in Table I disodium phenyl phosphate was the substrate. Table II shows that, in addition to phenyl phosphate, the enzyme was also able to hydrolyze either α-glycerophosphate or β-glycerophosphate. In each instance the substrate concentration was optimal. The enzyme hydrolyzed phenyl phosphate more rapidly than either of the glycerophosphates, and β-glycerophosphate more rapidly than α-glycerophosphate. This has also been observed for the alkaline phosphatases of other tissues (12, 35, 40, 42, 51). The optimum pH for each of these substrates is slightly different. In this respect the work of King and Delory (42) is important, for they found that the rate of hydrolysis of these three substrates was in the same order, even if measurements were made at the optimum pH for each.

TABLE II

HYDROLYSIS OF DIFFERENT SUBSTRATES BY RABBIT POLYMORPHONUCLEAR LEUCOCYTE ALKALINE PHOSPHATASE. INCUBATION TIME, 1 HR. TEMPERATURE, 37° C.

Substrate	Concentration	Phosphatase activity (mgm. phosphorus/100 ml. enzyme/hr.)		
		Preparation *A* (cell suspension)	Preparation *B* (saponin supernatant)	Preparation *C* (cell suspension)
Disodium phenyl phosphate	0.004 *M*	2.76	5.78	19.7
Sodium α-glycerophosphate	0.02 *M*	0.68	1.80	9.6
Sodium β-glycerophosphate	0.02 *M*	0.87	2.11	10.0

Enzyme Concentration

Fig. 1 shows that, under the conditions of the study, the extent of the hydrolysis of phenyl phosphate was proportional to the concentration of the enzyme.

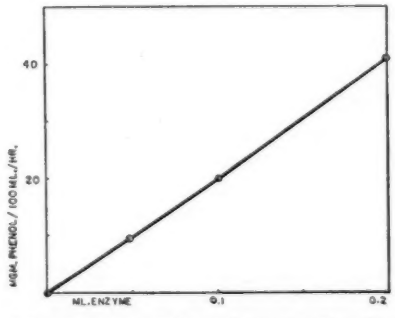

FIG. 1. *The relation between enzyme activity and concentration of rabbit polymorphonuclear leucocyte alkaline phosphatase. Substrate, disodium phenyl phosphate 0.004 M. Incubation time, 1 hr. Temperature, 37°C.*

Substrate Concentration

The effect of substrate concentration on the fully activated enzyme system is shown in Fig. 2, which gives the results of a typical experiment. The optimum substrate concentration was in the region of 0.004 *M*, concentrations greater than this being inhibitory. The Michaelis constant, K_s, for six different enzyme preparations with enzyme concentrations extending over a sixfold range, was evaluated by the method of Lineweaver and Burk (44). The figures varied from 0.00059 to 0.00096. Fig. 2 shows that in a typical experiment the Lineweaver and Burk (44) plot of $1/S$ against $1/V$ was reasonably linear. The pH was 9.9. These results can be compared with those of

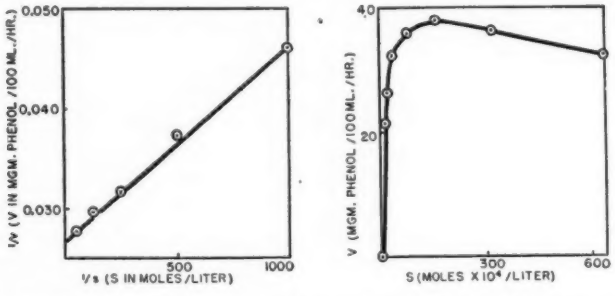

FIG. 2. *The effect of substrate concentration on the activity of rabbit polymorphonuclear leucocyte alkaline phosphatase. Substrate, disodium phenyl phosphate. Temperature, 37°C.*

Folley and Kay (21) who found a value of 0.0006 for mammary gland alkaline phosphatase at pH 10. Schmidt and Thannhauser (58), using a highly purified intestinal alkaline phosphatase, found that the K_s for phenyl phosphate was 0.000072 and 0.000078 for two different concentrations of the enzyme. However the study was made at pH 9.3, which is not the optimum for phenyl phosphate. Folley and Kay (21) found that the K_s of mammary gland phosphatase was 0.0001 instead of 0.0006 when measurements were made at pH 8.9 rather than pH 10, while Jacobsen (33) reported that the K_s of alkaline phosphatase was greatly affected by changes in pH. It thus appears probable that, had Schmidt and Thannhauser (58) determined the K_s at pH 10, they would have obtained a higher figure.

Martland and Robison (45), studying the hydrolysis of glycerophosphate by bone phosphatase, found no difference in enzyme activity when the substrate concentration ranged from 0.3 *M* to 0.003 *M*, a result confirmed by other workers when glycerophosphate was the substrate (8, 32, 58). Fig. 2 shows that this was not so for rabbit polymorphonuclear leucocyte phosphatase with phenyl phosphate as substrate; excess substrate was inhibitory, a result confirmed by Folley and Kay (21) for mammary gland phosphatase and Schmidt and Thannhauser (58) for intestinal phosphatase.

Haldane (30) suggested that the inhibition of an enzyme by excess of the substrate was due to one or more molecules of the substrate combining with

the dissociable enzyme–substrate complex to form a further addition compound, which does not break down to give the products of the reaction. Thus we have

$$E + S \rightleftharpoons ES \text{ (active)}$$
$$ES + S \rightleftharpoons ES_2 \text{ (inactive)}$$

where ES dissociates into the products of the reaction and ES_2 is inactive and does not dissociate. The constant of the first reaction is K_s and that of the second can be represented by K_2. A mathematical development of this theory gives

$$\frac{S}{V} = \frac{K_s}{V_M} + \frac{1}{V_M}\left(S + \frac{S}{K_2}\right)$$

where S is the concentration of the substrate, V_M is the maximum velocity and V the observed initial velocity of the hydrolysis (Lineweaver and Burk (44), Case III).

In one experiment, in which higher substrate concentrations than those of the experiment of Fig. 2 were used, K_2 was evaluated for the rabbit polymorphonuclear leucocyte alkaline phosphatase. The mean value was 0.5, but the inaccuracies of the procedure were such that it was impossible to test the validity of the Haldane theory in this instance.

Course of Reaction with Time

A number of experiments using different enzyme preparations, enzyme concentrations, or substrate concentrations showed that the course of the reaction was linear with time for the 60 min. incubation period investigated. This meant that, under the conditions of study, the amount of phenol liberated in one hour gave an adequate measure of the initial velocity of the reaction.

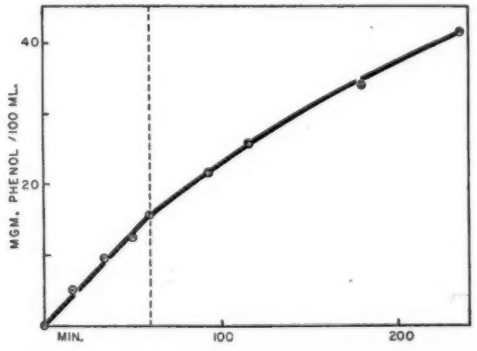

FIG. 3. *Time course of reaction for rabbit polymorphonuclear leucocyte alkaline phosphatase. Substrate, disodium phenyl phosphate 0.004 M. Temperature, 37° C.*

The same was true for other alkaline phosphatases (21, 32, 58). Fig. 3 represents the results of one of three experiments designed to show the effect of incubation periods longer than one hour. The results of the other experi-

TABLE III

COURSE OF REACTION WITH TIME. ENZYME, RABBIT POLYMORPHONUCLEAR LEUCOCYTE ALKALINE PHOSPHATASE. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 *M*. TEMPERATURE, 37° C.

	Time (hr.)	Phenol formed (mgm.)	Percentage hydrolysis	k $\left(\frac{2.303}{T}\log\frac{S}{S-X}\right)$
Experiment 1 (Saponin supernatant)	1	0.0299	1.59	0.0161
	2	0.0535	2.84	0.0146
	3	0.0695	3.71	0.0127
	4	0.0822	4.37	0.0112
Experiment 2 (Cell suspension)	1	0.0587	3.12	0.0320
	2	0.1105	5.88	0.0304
	3	0.1465	7.80	0.0273
	4	0.1745	9.28	0.0246

ments are given in Table III, which shows the amount of phenol liberated, the percentage hydrolysis of the substrate, and the first-order reaction constant calculated from:

$$k = \frac{2.303}{T} \log \frac{S}{S - X}$$

where S is the concentration of the substrate and X is the amount of substrate hydrolyzed in T hours. In each instance, k became less as the reaction proceeded. Since the substrate concentration was optimal, one would not expect the course of the hydrolysis of phenyl phosphate to simulate that of a first-order reaction. First order kinetics are usually not observed for enzymatic reactions until the substrate concentration has fallen considerably below the level necessary to "saturate" the enzyme. Table III shows that the rate of hydrolysis of phenyl phosphate fell off more rapidly than would be expected if the reaction were described in terms of the unimolecular law. This means that some explanation, other than substrate depletion, must be sought to explain the decrease in activity. It has been suggested (13, 45) that this progressive decrease in activity might be caused by the inhibitory effect of inorganic phosphate formed during the course of the reaction.

Phosphate Inhibition

Table IV shows that 0.01 *M* phosphate greatly inhibited the enzyme and that concentrations as low as 0.0003 *M* caused an observable inhibition. This would favor the suggestion (45) that the falling off in activity faster than would be expected by the unimolecular law was due to phosphate inhibition. If, as in Experiment 2, Table III, hydrolysis had proceeded to the extent of 10% of the substrate, the phosphate concentration would be 0.0004 *M*, a concentration in excess of 0.0003 *M*, which was definitely inhibitory (Table IV).

The effect of phosphate on other alkaline phosphatases has also been described (45, 53, 58). By far the most extensive study was that of Jacobsen

TABLE IV

EFFECT OF PHOSPHATE ON RABBIT POLYMORPHONUCLEAR LEUCOCYTE ALKALINE PHOSPHATASE. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 *M*. INCUBATION TIME, 1 HR. TEMPERATURE, 37° C.

Phosphate concentration	Preparation 1 (cell suspension)		Preparation 2 (cell suspension)		Preparation 3 (alkyl sulphate supernatant)	
	Mgm. phenol /100 ml. /hr.	Relative activity in presence of PO^4, %	Mgm. phenol /100 ml. /hr.	Relative activity in presence of PO_4, %	Mgm. phenol /100 ml. /hr.	Relative activity in presence of PO_4, %
0	2.8	100	17.7	100	25.8	100
0.00031 *M*	2.4	86	15.6	88		
0.00062 *M*	2.5	89	15.9	90		
0.00125 *M*	2.1	75	15.0	85		
0.0025 *M*	2.0	72	13.2	75		
0.005 *M*	1.8	64	10.5	59		
0.010 *M*	1.4	50	8.1	46	12.7	49

(32), who showed that the inhibition of kidney alkaline phosphatase by phosphate was partly competitive and partly noncompetitive, i.e. independent of substrate concentration.

Hydrogen Ion Concentration

Many of the earlier workers on alkaline phosphatase reported that the optimum pH was in the region of 9 (35, 36, 38, 52, 53). It is now known that the optimum pH of alkaline phosphatase depends upon both the nature (42) and the concentration (21, 33) of the substrate. Also true initial velocities must be measured; that this has not always been done may be the reason for many of the discrepancies found in the literature.

Fig. 4 shows the pH activity curve of rabbit polymorphonuclear phosphatase using the "universal buffer" of Prideaux and Ward (49). The optimum is at pH 10. Using borate, diethylbarbiturate, or glycine buffers, the optimum pH was, in each instance, between 9.75 and 10.0. This agrees with the results of Folley and Kay (21), who found the optimum pH of mammary gland phosphatase to be 10 with 0.0045 *M* phenyl phosphate as substrate, and King and Delory (42), who found the optimum pH of intestinal phosphatase with 0.002 *M* phenyl phosphate to be 9.76.

Many of the buffers commonly used for determining the effect of pH on enzyme systems inhibit the alkaline phosphatase of rabbit polymorphonuclear leucocytes. Table V shows the extent of this inhibition with each of the four buffers used. In each instance the pH was adjusted to 9.9 before the test and the concentration of buffer was the same as that used in determining the optimum pH of the enzyme. That diethylbarbiturate inhibited bone alkaline phosphatase considerably less than the same concentration of glycine has been

reported previously (2). For all subsequent work on the effect of pH on the enzyme activity, a modification of the Michaelis (46) diethylbarbiturate buffer, described by King and Delory (42), was used.

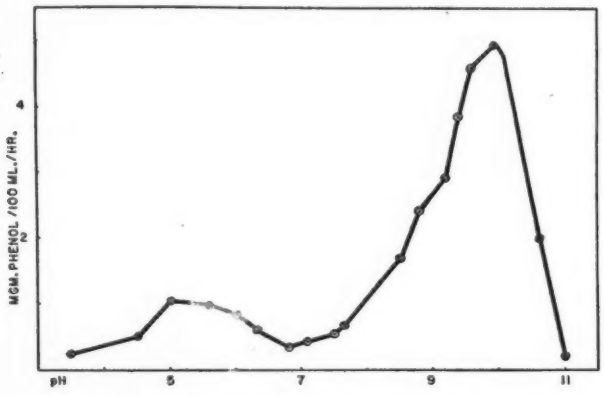

FIG. 4. *The effect of hydrogen ion concentration on the activity of rabbit polymorphonuclear leucocyte alkaline phosphatase. Substrate, disodium phenyl phosphate 0.004 M. Incubation time, 1 hr. Temperature, 37° C.*

TABLE V

EFFECT OF VARIOUS BUFFERS ON RABBIT POLYMORPHONUCLEAR LEUCOCYTE ALKALINE PHOSPHATASE. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 *M*. INCUBATION TIME, 1 HR. TEMPERATURE, 37° C.

Buffer	Phosphatase activity (mgm. phenol/100 ml./hr.)		Relative activity in presence of additional buffer, %
	No additional buffer	Additional buffer	
Borate	9.6	6.8	71
Universal buffer	10.2	5.6	55
Diethyl-barbiturate	7.4	6.9	93
Glycine	21.1	8.8	42

Fig. 5 shows the effect of substrate concentration on the pH optimum. With lower substrate concentrations the optimum pH was lower, a result reported for other alkaline phosphatases (21, 33).

The pH activity curve of the phosphatases from many tissues has two maxima, one in the neighborhood of 5 and one between 9 and 10 (3, 4, 12, 48). It is now generally conceded that there are at least two different phosphatases in most tissues and Perlmann and Ferry (48) have succeeded in separating the phosphatase of kidney into two fractions, one active at low and one at high pH values. The rabbit polymorphonuclear leucocyte phosphatase, with maxima in the region of pH 5 and pH 10, is thus similar to the phosphatases of other tissues.

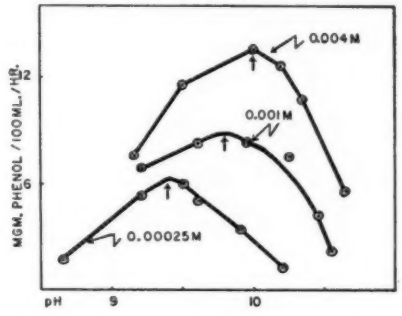

FIG. 5. *The effect of substrate concentration on the optimum hydrogen ion concentration of rabbit polymorphonuclear leucocyte alkaline phosphatase. Substrate, disodium phenyl phosphate. Incubation time, 1 hr. Temperature, 37° C.*

Magnesium

The activity of the rabbit polymorphonuclear leucocyte phosphatase was increased greatly in the presence of magnesium ions. Table VI shows that the mean activity in the presence of 0.001 *M* magnesium was approximately three times that of the unactivated enzyme. The optimum concentration

TABLE VI

EFFECT OF MAGNESIUM IONS ON RABBIT POLYMORPHONUCLEAR LEUCOCYTE ALKALINE PHOSPHATASE. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 *M*. INCUBATION TIME, 1 hr. TEMPERATURE, 37° C.

Phosphatase activity (mgm. phenol/100 ml./hr.)		Relative activity in presence 0.001 *M* Mg^{++}, %
No Mg^{++}	0.001 *M* Mg^{++}	
53.4	210.0	392
13.2	44.1	330
10.7	18.3	175
10.5	22.8	217
9.3	30.1	323
9.0	26.6	295
9.0	24.6	274
5.8	17.2	296
3.8	11.9	313
3.6	11.7	321
2.9	5.8	200
2.3	9.1	391
1.3	3.9	300
	Mean (± S.d.) =	302.1 (± 63.0)

of magnesium was 0.001 *M* (Fig. 6). This effect of magnesium ions on alkaline phosphatase activity is well known and will be discussed in greater detail in a subsequent paper.

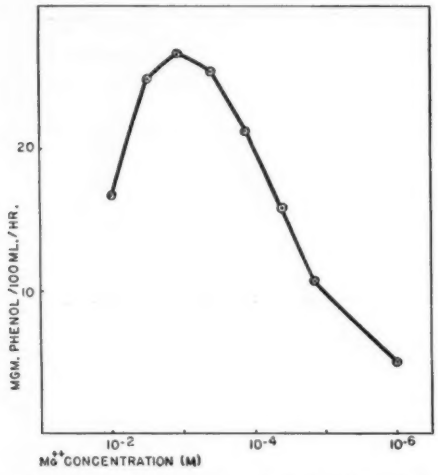

FIG. 6. *The effect of magnesium ions on the activity of rabbit polymorphonuclear leucocyte alkaline phosphatase. Substrate, disodium phenyl phosphate 0.004 M. Incubation time, 1 hr. Temperature, 37°C.*

Surface-active Substances

It was reported by Rossiter (56) and Cram and Rossiter (11) that surface-active substances, such as saponin, alkyl sulphate,* or bile salts, liberated alkaline phosphatase from the rabbit polymorphonuclear leucocyte. Table VII shows the effect of these substances in concentrations that were found to be optimal. The effect of sodium taurocholate was less than that of saponin or alkyl sulphate for, as will be shown in the following section, this concentration of bile salt was itself inhibitory to cell-free preparations of the enzyme.

TABLE VII

EFFECT OF SURFACE-ACTIVE SUBSTANCES ON THE LIBERATION OF ALKALINE PHOSPHATASE FROM SUSPENSIONS OF WASHED RABBIT POLYMORPHONUCLEAR LEUCOCYTES. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 *M*. MAGNESIUM CONC., 0.001 *M*. INCUBATION TIME, 1 HR. TEMPERATURE 37° C.

Cells suspended in:	Alkaline phosphatase activity (mgm. phenol/100 ml./hr.)	
	Suspension *A*	Suspension *B*
Isotonic saline	5.7	16.8
1% Saponin in isotonic saline	12.3	57.1
0.0625% Alkyl sulphate in isotonic saline	12.4	58.8
0.00625 *M* sodium taurocholate in isotonic saline	10.6	50.0

* "*Purified alkyl sulfate*" *provided by the Procter and Gamble Company, Cincinnati, Ohio.*

Inhibitors

Bile salts, glycine, zinc, or cyanide were found to inhibit the alkaline phosphatase of the rabbit polymorphonuclear leucocyte, while fluoride was without effect.

Bile Salts

Whereas bile salts increased the activity of suspensions of cells by liberating the enzyme into the surrounding fluid, the effect on cell-free preparations was invariably inhibitory (Table VIII). Bodansky (7) found that bone,

TABLE VIII

EFFECT OF BILE SALTS ON CELL-FREE PREPARATIONS OF RABBIT POLYMORPHONUCLEAR LEUCOCYTE ALKALINE PHOSPHATASE. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 M. INCUBATION TIME, 1 HR. TEMPERATURE 37°C. 1% SAPONIN SUPERNATANT

Bile salt	Concentration	Phosphatase activity (mgm. phenol/100 ml. enzyme /hr.)		Relative activity in presence of bile salt, %
		No bile salt	Bile salt	
Sodium desoxycholate	0.00125 *M*	27.3	24.2	89
Sodium desoxycholate	0.00125 *M*	21.6	17.4	81
Sodium taurocholate	0.00125 *M*	27.3	23.4	86
Sodium taurocholate	0.00125 *M*	21.6	18.9	87
Sodium taurocholate	0.00125 *M*	60.0	51.0	85
Sodium taurocholate	0.00625 *M*	60.0	38.7	65

kidney, or serum alkaline phosphatase was reduced to 65 to 75% of its activity in the presence of 0.00125 *M* sodium desoxycholate and to 45 to 55% of its activity in the presence of 0.00625 *M* sodium taurocholate while intestinal phosphatase was not inhibited. This result has recently been confirmed (55). The white cell enzyme was inhibited to 80 to 90% of its activity in the presence of 0.00125 *M* sodium desoxycholate and to 65% of its activity in the presence of 0.00625 *M* sodium taurocholate, thus resembling the phosphatase of bone, kidney, or serum rather than that of the intestinal mucosa.

Glycine

Table IX shows that in the presence of low concentrations of glycine (optimum 0.005 *M*) the activity of the enzyme was increased, but that in the presence of high concentrations of glycine the enzyme was inhibited. This activating effect of low concentrations of glycine has frequently been reported for the phosphatases of other tissues (5, 6, 62), but Schmidt and Thannhauser (58) found that 0.0015 *M* glycine had no effect on a purified intestinal phosphatase. The inhibitory action of high concentrations of glycine is also well known (5, 6, 8, 62).

TABLE IX

EFFECT OF GLYCINE ON RABBIT POLYMORPHONUCLEAR LEUCOCYTE ALKALINE PHOSPHATASE. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 *M*. INCUBATION TIME, 1 HR. TEMPERATURE, 37° C.

Glycine concentration	Preparation 1 (cell suspension)		Preparation 2 (cell suspension)	
	Mgm. phenol /100 ml./hr.	Relative activity in presence of glycine, %	Mgm. phenol /100 ml./hr.	Relative activity in presence of glycine, %
0	5.7	100	13.1	100
0.00007 *M*	6.0	105	12.6	96
0.00021 *M*	5.9	103	12.5	95
0.00062 *M*	7.1	124	14.5	111
0.00186 *M*	7.7	135	17.6	134
0.00557 *M*	8.0	140	19.5	149
0.0167 *M*	8.0	140	18.3	140
0.05 *M*	7.4	130	18.3	140
	Preparation 3 (saponin supernatant)		Preparation 4 (saponin supernatant)	
	Mgm. phenol /100 ml./hr.	Relative activity in presence of glycine, %	Mgm. phenol /100 ml./hr.	Relative activity in presence of glycine, %
0	27.3	100	21.6	100
0.001 *M*	27.5	101	24.3	112
0.01 *M*	24.3	89	21.0	97
0.1 *M*	8.0	29	9.0	42

Zinc

Very low concentrations of zinc ion (10^{-6} *M*) inhibited the enzyme (Table X). Other alkaline phosphatases are progressively inhibited by increasing concentrations of zinc ions (14, 31, 48).

Cyanide

Table XI shows that low concentrations of cyanide (0.0001 *M* and 0.001 *M*) increased the activity of the enzyme, but that concentrations of 0.01 *M* were greatly inhibitory. Cloetens (9, 10) on the basis of cyanide inhibition, described two alkaline phosphatases: Phosphatase I, which was inactive in the absence of magnesium ions and not inhibited by 0.01 *M* cyanide, and Phosphatase II, which was affected variously by magnesium and inhibited by 0.01 *M* cyanide. By measuring the phosphatase activity in the presence of optimal concentrations of magnesium ions and in the presence or absence of 0.01 *M* cyanide, he was able to determine the ratio of Phosphatase I to Phosphatase II in various tissues. For intestine, serum, or bone the ratio was 0 to 14%; for kidney it was 8 to 50% and for liver it was 16 to 90%.

TABLE X

EFFECT OF ZINC ON RABBIT POLYMORPHONUCLEAR LEUCOCYTE ALKALINE PHOSPHATASE. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 *M*. INCUBATION TIME, 1 HR. TEMPERATURE 37° C.

Zinc concentration	Preparation 1 (cell suspension)		Preparation 2 (cell suspension)		Preparation 3 (saponin supernatant)	
	Mgm. phenol /100 ml. /hr.	Relative activity in presence of Zn^{++}, %	Mgm. phenol /100 ml. /hr.	Relative activity in presence of Zn^{++}, %	Mgm. phenol /100 ml. /hr.	Relative activity in presence of Zn^{++}, %
0	15.3	100	7.1	100	60.0	100
10^{-6} *M*	10.7	70	6.1	85	60.0	100
10^{-5} *M*	5.3	34	3.4	48	30.9	52
10^{-4} *M*	3.6	23	2.8	39	5.7	11
10^{-3} *M*	3.2	21	2.0	28	4.2	7

TABLE XI

EFFECT OF CYANIDE ON RABBIT POLYMORPHONUCLEAR LEUCOCYTE ALKALINE PHOSPHATASE. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 *M* OR SODIUM β-GLYCEROPHOSPHATE 0.02 *M*. INCUBATION TIME, 1 HR. TEMPERATURE, 37° C.

Cyanide concentration	Preparation 1 (cell suspension)		Preparation 2 (cell suspension)		Preparation 3 (cell suspension)	
	Mgm. phenol /100 ml. /hr.	Relative activity in presence of cyanide, %	Mgm. phenol /100 ml. /hr.	Relative activity in presence of cyanide, %	Mgm. phosphorus /100 ml. /hr.	Relative activity in presence of cyanide, %
0	9.0	100	8.0	100	1.2	100
10^{-4} *M*	12.2	135	10.7	133	2.0	167
10^{-3} *M*	11.7	129	11.1	138	1.6	133
10^{-2} *M*	1.8	20	1.2	15	0.15	13

Table XII shows that the rabbit polymorphonuclear leucocyte alkaline phosphatase was greatly inhibited by 0.01 *M* cyanide so that, in this respect, it resembled the phosphatase of intestine, serum, or bone rather than that of liver.

Fluoride

Low concentrations (0.0001 *M*) of fluoride ion had no effect on the enzyme, but 0.01 *M* fluoride increased the activity of the enzyme slightly (Table XIII). Although the alkaline phosphatase of kidney is not inhibited by fluoride, the phosphatase of yeast, active at pH 6.2, is greatly inhibited (1). In addition, Cloetens (10) found that Phosphatase I was inhibited in the presence of 0.01 *M* fluoride, but that Phosphatase II was not affected. Thus, on a basis of fluoride insensitivity as well as of cyanide sensitivity, the alkaline phosphatase of the

TABLE XII

EFFECT OF CYANIDE ON RABBIT POLYMORPHONUCLEAR LEUCOCYTE ALKALINE PHOSPHATASE. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 *M* OR SODIUM GLYCEROPHOSPHATE 0.02 *M*. MAGNESIUM CONCENTRATION, 0.01 *M*. INCUBATION TIME, 1 HR. TEMPERATURE, 37° C. FINAL CONCENTRATION OF CYANIDE, 0.01 *M*

Preparation	Substrate	Phosphatase activity (mgm. phenol (or P.) /100 ml./hr.)		Relative activity in presence of cyanide, %
		No cyanide	Cyanide 0.01 *M*	
Cell suspension	Disodium phenyl phosphate	9.0	1.8	20
Cell suspension	Disodium phenyl phosphate	8.0	1.2	15
Cell suspension	Sodium β-glycero-phosphate	1.2	0.15	13
Cell suspension	Disodium phenyl phosphate	30.6	3.9	12
Saponin supernatant	Disodium phenyl phosphate	26.9	1.4	5
Cell suspension	Sodium α-glycero-phosphate	6.9	2.7	39
Cell suspension	Sodium β-glycero-phosphate	7.6	2.1	28

rabbit polymorphonuclear leucocyte can be considered to be a Phosphatase II of Cloetens. The enzyme is not the specific hexose diphosphatase described by Gomori (26), since that enzyme is inhibited by fluoride and activated by cyanide.

TABLE XIII

EFFECT OF FLUORIDE ON RABBIT POLYMORPHONUCLEAR LEUCOCYTE ALKALINE PHOSPHATASE. SUBSTRATE, DISODIUM PHENYL PHOSPHATE 0.004 *M*. INCUBATION TIME, 1 HR. TEMPERATURE, 37° C.

Fluoride concentration	Preparation 1		Preparation 2	
	Mgm. phenol /100 ml./hr.	Relative activity in presence of fluoride, %	Mgm. phenol /100 ml./hr.	Relative activity in presence of fluoride, %
0	10.0	100	7.7	100
10^{-4} *M*	10.0	100	8.0	104
10^{-3} *M*	10.0	100	7.7	100
10^{-2} *M*	10.4	104	9.0	117

Discussion

To compare the alkaline phosphatase content of one tissue with that of another is difficult because, in addition to the uncertainties of extraction already mentioned, each worker has usually employed his own methods and has expressed his results in a variety of units. However, the figure of 52 King–Armstrong units (43) per gm. fresh tissue for the rabbit polymorphonuclear

leucocyte is of the same order as that reported by Folley and Greenbaum (19) for the alkaline phosphatase of both the kidney and the mammary gland of the rat. Expressed in the units employed in this study, the figures for the rat become 60 units per gm. fresh tissue for kidney and 7.5 units per gm. for resting mammary gland, rising to 30 units per gm. during lactation (20). Thus the concentration of alkaline phosphatase in the rabbit polymorphonuclear leucocyte is greater than that found in most other organs, for it is generally conceded that kidney and lactating mammary gland are two of the tissues of the body richest in alkaline phosphatase (22).

These experiments lend support to the suggestion of Roche (53) that the phosphatase of the white cell might be a source of the alkaline phosphatase of the serum. Fiessinger and Boyer (16), because of the low concentration of phosphatase that they found in white cells, considered this to be unlikely. We now know, however, that the concentration of alkaline phosphatase in the polymorphonuclear leucocyte of the rabbit is some 10 times greater than that reported by Fiessinger and Boyer (16). But the possibility still remains that the white cell may have obtained its phosphatase by adsorption of the enzyme from the plasma. If this were so, the white cells must have the extraordinary ability of concentrating the enzyme by a factor of 1000, for the concentration of alkaline phosphatase in the rabbit polymorphonuclear leucocyte is of the order of 5000 King–Armstrong units per 100 gm. packed white cells compared with 5 units per 100 ml. for rabbit plasma (Haight and Rossiter (29)).

It seems clear that the alkaline phosphatase is within the cell rather than adsorbed on the cell surface, for its presence has been demonstrated by a number of workers (15, 24, 34, 61, 63) using the histochemical techniques of Gomori (23) and Takamatsu (59). Gomori (25) also claimed that there was little acid phosphatase in polymorphonuclear leucocytes, a result that has subsequently been confirmed (50, 63, 64).

The alkaline phosphatase of the polymorphonuclear leucocyte of the rabbit resembles the alkaline phosphatase of bone or kidney in that it has a similar optimum pH, K_s , and sensitivity to inhibitors such as phosphate, high concentrations of glycine, bile salts, zinc ions, cyanide, or fluoride. It also resembles the bone and kidney phosphatase in that its activity is increased by magnesium ions or low concentrations of glycine. It differs from the alkaline phosphatase of the intestine in that it is inhibited by bile salts and differs from the principal alkaline phosphatase of the liver in that it is inhibited by cyanide and not by fluoride. The enzyme can be considered to belong to Class AI of Folley and Kay (22) and the acid phosphatase to Class AII. The alkaline phosphatase can also be classed as a Phosphatase II of Cloetens (9).

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